Hyperforin biosynthesis - Characterization and purification of a prenyltransferase from *Hypericum calycinum* cell cultures

Von der Fakultät für Lebenswissenschaften
der Technischen Universität Carolo-Wilhelmina
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zur Erlangung des Grades einer
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genehmigte
D i s s e r t a t i o n

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Publications

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Publications

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Presentations

-Short lecture

Boubakir, Z., Liu, B., Zapletalová, P., Beerhues, L. (2003)
Molecular analysis of the biosynthesis of the active compounds in *Hypericum perforatum.*
DPhG-Jahrestagung (Würzburg, 08.-11. Oktober 2003)

Boubakir, Z., Beerhues, L. (2005)
Purification of a prenyltransferase related to hyperforin biosynthesis
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-Poster

Boubakir, Z., Beuerle, T., Beerhues, L. (2004)
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Abbreviations

λ	wavelength
μ	micro
BDS	modified B5 medium by Dunstan and Short (1977)
CHT5-I	Ceramic Hydroxyapatite,Type I
CHAPS	3-[(3-cholamidopropyl) dimethylammonio-1-propane-sulfonate
DEAE	Diethylaminoethyl
DMAPP	Dimethylallylpyrophospate
DTT	1,4-Dithiothreitol
DTE	1,4-Dithioerythritol
FPLC	Fast protein liquid chromatography
FPP	Farnesylpyrophosphate
GC	Gas chromatography
GGPP	Geranylgeranylpyrophosphate
GPP	Geranylpyrophosphate
h	hours
HIC	Hydrophobic Interaction Chromatography
H. c	*Hypericum calycinum*
H. p	*Hypericum perforatum*
HPLC	High performance liquid chromatography
IEF	isoelectric focusing
IPG	Immobilized pH gradient
IPP	Isopentenylpyrophosphate
kat	Katal
kDa	Kilo Dalton
K_m	Michaelis constant
KPi	Potassium phosphate buffer
LB	Luria-Bertani
LS	Linsmeier and Skoog
M_r	Molecular mass
MS	Mass spectrum

m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
Q	Quaternary aminoethyl
rpm	Revolutions per minute
RP	Reversed Phase
SDS	Sodium dodecyl sulphate
TEMED	*N,N,N´,N´*-Tetramethylethylenediamine
THA	Trihydroxyacetophenone
Tris	Tris-(hydoxymethyl)aminomethan
w/v	Weight per volume
UV	Ultraviolet

1. Introduction

1.1. St. John’s wort extracts

Preparations from the aerial parts of St. John's wort (*Hypericum perforatum* L.) are used for the treatment of mild to moderate depression in many countries (Müller, 2003). The extracts are an accepted alternative to synthetic antidepressants and behavioural therapy. Thus, they are used in a therapeutic area which well extends beyond the traditional field of herbal medicine. St. John's wort extracts exhibit an inhibitory effect on the neuronal reuptake not only of serotonin, noradrenaline, and dopamine but also of gamma-aminobutyric acid (GABA) and L-glutamate. No other antidepressant shows an equally broad inhibitory profile.

Over the past years, a number of double-blind, randomized and controlled clinical studies were carried out which demonstrated the efficacy and good tolerability of St. John's wort extracts in mild to moderate depressive disorders (Müller, 2003). The therapeutic efficacy has only been questioned by a recent American study (Shelton et al., 2001). However, this clinical trial included patients with severe depression, an indication for which St. John’s wort extracts are not approved. Furthermore, a synthetic antidepressant used as a comparative drug also failed to be superior to placebo. So far, all clinical studies including the latter one confirmed the low frequency of adverse events caused by *Hypericum* preparations. However, a number of drug interactions have been found to occur with St. John's wort extracts, some of which are of clinical relevance (Müller, 2003).

Preparations from St. John's wort are among the most prescribed medicines in Germany (Lohse et al., 2000; Schulz et al., 2001). The numbers of prescriptions have approximately tripled since 1993. In 1999, around 130 million daily doses were prescribed. As a result of the health reform in Germany, the costs for *Hypericum* preparations are generally no longer reimbursed by the statutory health insurance providers, even though their benefit and safety have been unequivocally demonstrated. The costs for St. John's wort preparations are significantly lower than those for synthetic antidepressant medications. In the USA, St. John's wort extracts are increasingly used as an over-the-counter remedy for the treatment of depression. Some American medical societies recently included St. John's wort as an antidepressant in their guidelines on the drug treatment of depressive disorders.

The recommended minimum dosage for St. John's wort preparations is 500 mg per day of a 5:1 extract from the flowering tops and leaves. The minimum time of treatment is 4-6 weeks.

1.2. Depression

Depression is a common and potentially life-threatening (risk of suicide) disorder with an estimated life time prevalence of around 17% (Kessler et al., 1994). Its prevalence in Western industrialized nations is estimated to be approx. 3% in males and 7% in females (Depression Guideline Panel, 1993). Women experience depression about twice as often as men (Blelhar and Oren, 1995). Many hormonal factors may contribute to the increased rate of depression in women. The World Health Organization (WHO) estimates that the burden of depressive illness by the year 2020 will parallel morbidity and economic loss caused by heart disease (World Health Organization, 1998). Apart from the personal suffering of patients, depression is extremely expensive for health insurance systems, in terms of medication, medical consultation, days off work etc.

The major biological cause of depression is the deficiency in neurotransmitters which regulate mood and emotion, such as serotonin and noradrenaline (norepinephrine) (Müller, 2003). Antidepressant medications treat depression by restoring the normal levels of these two neurotransmitters released into the synaptic gap. Importantly, these acute effects are followed within 1-2 weeks by adaptive changes at the level of the receptors, e.g. up-regulation of α_1 and 5-HT_1 receptors and down-regulation of ß and 5-HT_2 receptors in the frontal cortex. These adaptive changes in the density of the receptors correlate with the time course of the antidepressant effect in patients.

The clinically used antidepressant drugs, e.g. the tricyclics imipramine and amitriptyline and the monoamine oxidase (MAO)-inhibitors phenelzine and tranylcypromine, were introduced more than forty years ago (Müller, 1997). Although powerful antidepressants, their therapeutically use was always accompanied by the burden of a high frequency of side effects. These problems led to the development of a number of second and third generation antidepressant drugs, such as reversible MAO-A inhibitors, specific serotonin reuptake inhibitors (SSRI), and specific serotonin and norepinephrine reuptake inhibitors. None of these newly developed antidepressants have conclusively shown to be superior to tricyclics in terms of antidepressive efficacy, but most of these newer compounds are better tolerated and

safer in overdose. Accordingly, in many western countries, the second generation compounds such as the SSRIs account for most of the antidepressant drug prescriptions.

1.3. *Hypericum perforatum* L.

Saint John's wort, also called common or perforate St. John's wort (Fig. 1-1), belongs to the family Clusiaceae (formerly Hypericaceae or Guttiferae). The species name "perforatum" is Latin for "perforated". The leaves of *H. perforatum*, when held to the light, reveal translucent dots, giving the impression of perforated leaves. The dots are large spherical glands spanning the whole leaf from the upper to the lower epidermis (Weiss, 1988). They contain essential oil comprising caryophyllene, pinenes, limonene, and myrcene as major components.
Besides the colorless glands, leaves contain dark red to black glands which accumulate hypericins. Especially the petals of the bright yellow-orange flowers are peppered with the black dots. When petals are rubbed between the fingers, a red sap is squeezed out and stains the fingers red. The stem of *H. perforatum* is unusual in that it has two raised lines making it appear pressed flat. Most plants have round or four-square stems (Weiss and Fintelmann, 2000).

St. John's wort is native to Europe and occurs throughout this continent, except the extreme North (Erdelmeier, 1998). In Asia, Africa, North and South America and Australia, it has been naturalized in waste places and along roadsides. It is one of those European native plants that have followed European settlers wherever they travelled in the world. The plant was introduced by early settlers to North America. In 1793, the first recorded specimen, grown without cultivation, was collected in Pennsylvania (Hahn, 1992).

Fig. 1-1: ***Hypericum perforatum*** **L.**

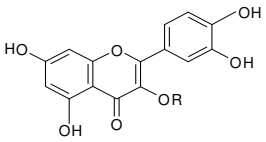
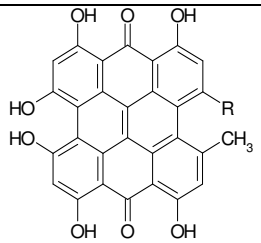
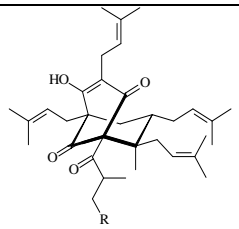
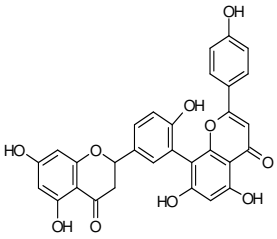
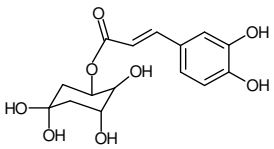
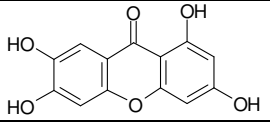
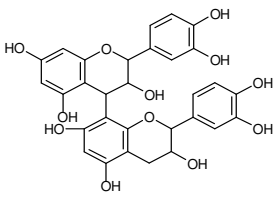
1.3.1 Constituents

St. John's wort is one of the best studied medicinal plants. It contains a complex mixture of secondary metabolites (Kurth and Spreemann, 1998). The main constituents are shown in Tab. 1-1 (Butterweck and Nahrstedt, 2003). The pharmacological activity of *Hypericum* preparations is attributed to hyperforins, hypericins and flavonoids (Simmen et al., 2001; Butterweck et al., 2001). The effectiveness of formulations to relieve depression depends on their hyperforin content (Laakmann et al., 1998).

Hypericins and flavonoids were found to have an effect in the Porsolt (forced swimming) test (Butterweck, 2003). These observations, however, were questioned by other authors (Müller, 2003). The biological evaluation of hypericins is hampered by their poor water solubility which, however, can be increased in the presence of procyanidins (Butterweck, 2003). Hypericins also show anti-inflammatory activity by inhibiting release of leukotrienes (Panossian et al., 1996) and prevent replication of encapsulated viruses in animal models (Meruelo et al., 1989).

Among the flavonoids are rutin, hyperoside, isoquercitrin, quercitrin and quercetin. The glucoside, galactoside and glucuronide of quercetin were active compounds in the Porsolt test, which was attributed to differences in the bioavailability of the glycosides (Butterweck, 2003). The aglycone quercetin was inactive in the forced swimming test. Besides their antidepressant properties, flavonoids were found to exhibit antioxidant activity (Sloley et al., 2000). This property was proposed to increase the overall extract efficacy by preventing oxidative degradation of other phytochemicals in *Hypericum* preparations.

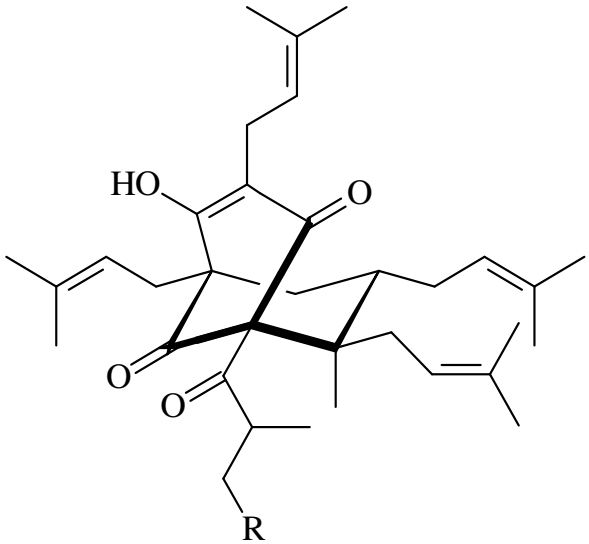
Tab. 1-1: Major constituents of St. John's wort

Group of compounds	Chemical structure	Individual names	Approx. content in plant extracts [%]
Flavonoids		R = H quercetin R = α-L-rhamnosyl quercitrine R = β-D-glucosyl isoquercitrin R = β-D-galactosyl hyperoside R = β-D-rutinosyl rutin R = α-D-glucuronyl miquelianin	0.2 - 0.6 0.2 - 0.4 0.5 - 0.8 2 - 4 2 - 3 0.4 - 0.5
Anthracene derivatives		R = CH_3 hypericin R = CH_2OH pseudohypericin	0.08 - 0.2 0.15 - 0.4
Prenylated phloroglucinols		R = H hyperforin R = CH_3 adhyperforin	0.9 - 5
Biflavones		I3', II8-biapigenin (amentoflavon) I3, II8-biapigenin	0.01- 0.05 0.2 - 0.6
Phenylpropanes (caffeic acid derivatives)		e.g. chlorogenic acid	< 1
Xanthones		1,3,6,7-tetrahydroxyxanthone	< 0.0004
Proanthocyanidins		e.g. procyanidines	Traces

1.4. Hyperforin

Hyperforin [$C_{35}H_{52}O_4$] (Fig. 1-2) is the major acylphloroglucinol in *H. perforatum*. Its content increases up to 5% of dry weight in flowers and leaves (Erdelmeier et al., 1998). The concentration of hyperforin changes during flower ontogenesis (Tekel'ovà et al., 2000). The content increased from 2.5% in the first developmental stages of buds to 6.6% in flowers just opened. In fruits, the amount even reached 8.1%.

Hyperforin was found in 1971 as the antibacterial principle of St. John's wort by Russian researchers (Gurevich et al., 1971). Hyperforin was shown to be active against gram-positive bacteria such as *Staphylococcus aureus*. There were earlier reports on the antibacterial properties of lipophilic extracts from *H. perforatum* (Neuwald and Hagenström, 1954). In the United States, a patent was granted in 1951 for the use of *H. perforatum* extracts as food preservative (Jensen, 1951).



Hyperforin R = H
Adhyperforin R = CH_3

Fig. 1-2: Chemical structure of hyperforins

Hyperforin is poorly stable when extracted from the herb and exposed to light and air (Erdelmeier, 1998; Bilia et al., 2001). Its liability to oxidation complicates the preparation of pharmaceutical and nutritional formulations of *H. perforatum* extracts (Guilhermano et al., 2004). The pharmacological activities may be rapidly lost during storage.

1.4.1 Pharmacology

Hyperforin inhibits the reuptake of serotonin, noradrenaline, dopamine, GABA (gamma-aminobutyric acid) and L-glutamate to similar extents, which is in clear contrast to the properties of all other known antidepressants. The combination of these effects gives it antidepressive and sedative properties (Teufel and Gleitz, 1997). The research has challenged this belief, focusing on hyperforin (Calapai et al., 1999). It is not only the broad-spectrum activity but also the underlying mode of action that is unique to hyperforin. The compound does not directly interact with the transmitter transporters but elevates the intracellular sodium concentration, thereby inhibiting the gradient-driven neurotransmitter reuptake (Singer et al., 1999). This is a novel mechanism of action which explains the broad-band reuptake inhibitory activity. Synthetic antidepressants are competitive inhibitors of one or maximally two transporters at the transmitter binding sites. Among all individual constituents of St. John`s wort, hyperforin is the only neurotransmitter reuptake inhibitor.

Besides uptake inhibition, hyperforin has the ability to inhibit *in vivo* the growth of human and rat tumour cell lines by induction of apoptosis (Schempp et al., 2002). In addition, it was shown to display antibacterial properties, explaining the traditional use of St. John's wort extracts for the local treatment of infected wounds (Hubner, 2003).

Hyperforin is also a potent ligand for the pregnane X receptor which regulates the expression of CYP 3A4 which in turn is involved in the oxidative metabolism of >50 % of all drugs (Moore et al., 2000). This can lead in the case of co-medication to a reduction of the plasma levels of other medications. Relevant interactions occur with drugs such as cyclosporin and protease inhibitors, resulting in a clear contraindication for St. John`s wort.

1.5. Prenyltransferases

A biosynthetic pathway of hyperforin was proposed on the basis of retrobiosynthetic analyses (Adam et al., 2002). The structure of hyperforin is derived from an acylphloroglucinol skeleton which is expanded into a bicyclo[2,2,1]nonaendionol, substituted with several lipophilic isoprenoid chains. The enzymes involved in prenylation and utilization of isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) are deemed prenyltransferases in accordance with the terminology proposed by Holloway and Popjàk (1967). Prenyltransferases are classified as follows:

1. Prenyl-prenyltransferases (chain-elongating prenyltransferases): Enzymes that (a) transfer IPP units to allylic diphosphates such as DMAPP, GPP and FPP in a head-to-tail fashion or (b) condense C_{15} or C_{20} prenylpyrophosphates in a tail-to-tail fashion.

2. Prenyl-aryltransferases (aromatic prenyltransferases): Enzymes that transfer (a) a mono-prenyl group or (b) a polyprenyl group to an aryl moiety.

Prenyltransferases generally mediate electrophilic reaction mechanisms involving carbocationic intermediates, a feature of terpenoid biochemistry (Croteau et al. 2000). The resonance-stabilized carbocation, generated by the pyrophosphate leaving group, can react with an extender substrate such as IPP.

Prenylation of aromatic substrates is involved in the biosynthesis of diverse molecules that play important roles in bacteria to mammals, e.g. electron transport via ubiquinone and plastoquinone (Grunler et al., 1994). In higher plants, prenylation reactions highly contribute to the diversification of aromatic secondary metabolites, with regard to both their chemical structures and biological activities. For instance, some prenylated flavonoids act as phytoalexins that are involved in plant defence mechanisms (Tahara and Ibrahim, 1995; Morrandi, 1996). Furthermore, they were reported to be potential natural medicines (Henderson et al., 2000; Miranda et al., 2000)

1.6. *Hypericum calycinum* L.

Recently, the formation of hyperforin and its homologue adhyperforin has been observed in cell cultures of *Hypericum calycinum* which are thus a valuable *in vitro* system for studying hyperforin biosynthesis (Klingauf et al., 2005).

Fig.1-3: ***Hypericum calycinum*** **L.**

So far, hyperforins have not been detected in differentiated plants of *H. calycinum*, although several dearomatized polyprenylated acylphloroglucinols have been isolated from this species. These compounds accumulate in those parts of petals which are exposed to the outside in the unopened bud. This is the first time that constituents other than flavonoids were found to serve as floral UV pigments. Interestingly, the polyprenylated compounds also accumulate to a high extent in the anthers and ovarian wall and provide protection against herbivores. Thus, they fulfill both attractive and defensive functions in *H. calycinum* flowers (Decosterd et al., 1998; Gronquist et al., 2001).

1.7. Research strategies and objectives

The biosynthesis of hyperforins starts with a polyketide synthase reaction giving acylphloroglucinols. This aromatic intermediate undergoes a series of prenylation reactions, accompanied by an intramolecular cyclization to yield the bicyclic molecule. The goal of the present work was to study at the enzyme and gene levels the prenyltransferases participating in hyperforin biosynthesis (Fig. 1-4). The experiments were carried out with cell cultures of *H. calycinum*. The focus was on the enzyme that catalyzes the first prenylation step.

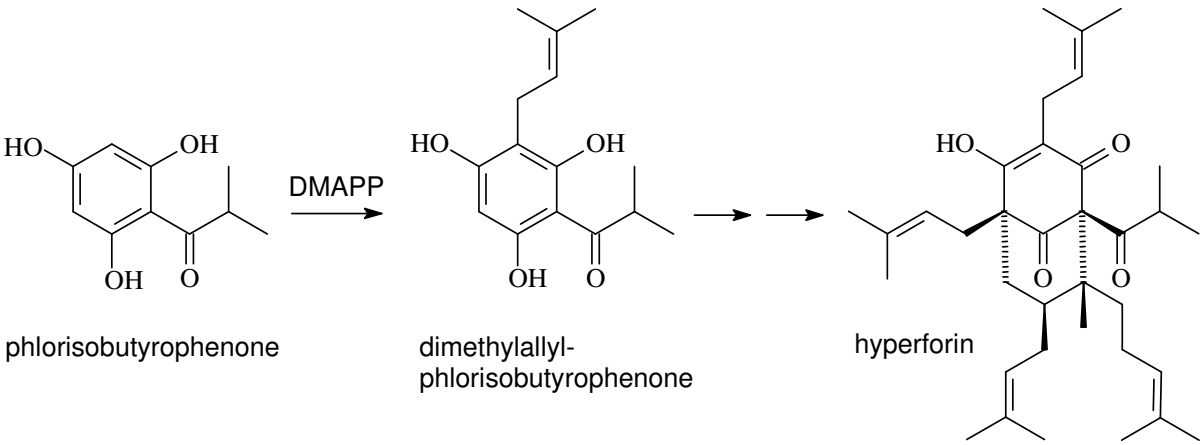


Fig. 1-4: The first prenylation step in hyperforin biosynthesis

2. Materials

2.1. Chemicals, nutrient media, solutions, buffers, equipment

2.1.1 Chemicals

Commonly used chemicals and gases were purchased in standard grade from the following companies:

Aldrich	Fluka	Roth
Applichem	Merck	Serva
Bio-Rad	Riedel-deHaën	Sigma

Aqueous solutions were prepared with milli-Q water that was purified by using the Milli-Q Water Purification System (Millipore).

Special chemicals

A. Enzyme assay

2,4,6-Trihydroxyacetophenone	Acros Organics
Phloroglucinol	Fluka
Phlorbenzophenone	ICN
Naringenin	Sigma
DTT (1,4-Dithiothreitol)	AppliChem
DTE (1,4-Dithioerythritol)	Roth
Isopentenyl diphosphate	Sigma
Geranyl diphosphate	Sigma
Farnesyl diphosphate	Sigma
Geranylgeranyl diphosphate	Sigma
DMSO	Fluka

B. Enzyme purification

Ammonium sulphate	Roth
Protein Molecular Weight Ladder	Fermentas
PD10-Sepharose G-25	Amersham Pharmacia Biotech

C. Gel Electrophoresis

Molecular weight marker kit	Sigma
Acrylamide/bis-acrylamide	Bio-Rad
Ammonium persulfate (APS)	Bio-Rad
Bromphenonl Blue	Aldrich
Coomassie Brilliant blue G250	Merck
Sodium dodecyl sulphate (SDS)	Roth
TEMED *(N, N, N´, N´*-Tetramethylethylenediamine)	Bio-Rad
Iodoacetamide	Acros Organics
Ampholyte	Bio-Rad
ReadyStrip^{TM} IPG Strip	Bio-Rad
Agarose, NEE Ultra Quality	Roth
Blotting filter paper	Schleicher & Schuell
Formaldehyde, 37%	Roth

2.1.2 Nutrient media for plant tissue cultures

Media were prepared as described below (Tab. 3-1). Aliquots of 50 ml were filled into 300 ml Erlenmeyer flasks, sealed with aluminium caps and sterilized by autoclaving at 120°C for 20 minutes.

Tab. 3-1: Stock solutions and preparation of media

Medium	Composition		Preparation and use
BDS-Liquid medium (modified B5 medium; Gamborg et al., 1968; Dunstan and Short, 1977)	**I**		Mix with 800 ml distilled water, add and dissolve saccharose, adjust the volume to one liter with water and the pH to 5.5. Autoclave Nutrient medium for cell suspension cultures
	$CaCl_2 \cdot 2H_2O$	150 mg/l	
	KNO_3	2530 mg/l	
	NH_4NO_3	320 mg/l	
	$NH_4H_2PO_4$	230 mg/l	
	$(NH_4)_2SO_4$	134 mg/l	
	$MgSO_4 \cdot 7H_2O$	247 mg/l	
	$MnSO_4 \cdot 4H_2O$	13.2 mg/l	
	$ZnSO_4 \cdot 7H_2O$	2.0 mg/l	
	II		
	$CuSO_4 \cdot 5H_2O$	0.039 mg/l	
	KI	0.75 mg/l	
	$CoCl_2 \cdot 6H_2O$	0.025 mg/l	
	$Na_2MoO_4 \cdot 2H_2O$	0.25 mg/l	
	H_3BO_3	3.0 mg/l	
	$NaH_2PO_4 \cdot 2H_2O$	172 mg/l	
	III		
	$FeSO_4$	27.85 mg/l	
	TitriplexIII ($Na_2EDTA \cdot 2H_2O$)	37.25 mg/l	
	IV		
	Nicotinic acid	1.0 mg/l	
	Thiamine hydrochloride	10.0 mg/l	
	Pyridoxal hydrochloride	1.0 mg/l	
	myo-Inositol	100.0 mg/l	
	V		
	Kinetin (6-Furfurylaminopurine)	100 mg/l	
	Dicamba (3,6-Dichloro-2-methoxybenzoic acid)	7.5 mg/l	
	VI		
	Sucrose	30 g/l	

LS-Liquid medium (Linsmeier and Skoog, 1965)	**I** KNO_3 1900 mg/l NH_4NO_3 1650 mg/l $CaCl_2 \cdot 2H_2O$ 440 mg/l $MgSO_4 \cdot 7H2O$ 370 mg/l KH_2PO_4 170 mg/l **II** $MnSO_4 \cdot H_2O$ 16.90 mg/l $ZnSO_4 \cdot 7H_2O$ 10.60 mg/l KI 0.83 mg/l H_3BO_3 6.20 mg/l $Na_2MoO_4 \cdot 2H_2O$ 0.25 mg/l $FeSO_4 \cdot 7H_2O$ 27.80 mg/l $CuSO_4 \cdot 5H_2O$ 0.025 mg/l $CoCl_2 \cdot 6H_2O$ 0.025 mg/l **III** Titriplex III (Na_2 EDTA $\cdot$ $2H_2O$) 41.30 mg/l **IV** myo–Inositol 100.0 mg/l Thiamine hydrochloride 0.4 mg/l **V** 2,4-Dichlorophenoxy-acetic acid 0.22 mg/l 1-Naphtylacetic acid 0.186 mg/l **VI** Sucrose 30 g/l	Mix with 800 ml distilled water, add and dissolve saccharose, adjust the volume to one liter with water and the pH to 6-6.3 with concentrated NaOH solution. Autoclave Nutrient medium for cell suspension cultures.
LS-Solid medium	See LS-Liquid medium, but add 8.0 g/l SELECT agar and heat the medium to nearly 80 °C	Nutrient medium for callus cultures

2.1.3 Solutions, buffers for biochemical analysis

Tab. 3-2: Buffers for extraction and enzyme incubation

Name	composition	Preparation and storage
Extraction buffer 0.1 M potassium phosphate pH 6.5 + 0.1 mM DTT	Potassium dihydrogen phosphate 1.36 g DTT 15.4 mg Water ad 100 ml	pH adjusted by KOH, stored at 4°C DTT is added freshly
Incubation buffer 0.1 M Tris-HCl buffer pH 7.5	Tris 1.21 g Water ad 100 ml	pH adjusted by concentrated HCl, stored at 4°C

Tab. 3-3: Solution for regeneration of PD_{10}- columns (Amersham Biosciences)

NaOH Cleaning agent	NaOH 0.16 M	Wash the column with five column volumes of 0.16 M NaOH and then five column volumes of distilled water

Tab. 3-4: Solution for protein estimation

Bradford-dye solution	Coomassie®-Brilliant blue G-250 100 mg Ethanol 96 % 50 ml Orthophosphoric acid 100 ml Water ad 1000 ml	Dissolve Coomassie® -Brilliant blue G-250 in ethanol, add orthophosphoric acid and complete the volume with water. The solution should be stored in a refrigerator and filtered before use

2.1.4 Buffers for protein purification

Tab. 3-5: Buffers for DEAE and Mono-Q anion exchange columns

Starting Buffer pH 7.5	20 mM Tris 1 mM DTT Water	0.242 g 150 µg ad 100 ml	Filter, degas and store at 4°C, pH adjusted by concentrated HCl
Elution Buffer pH 7.5	20 mM Tris 0.5 M KCl 1 mM DTT Water	0.242 g 3.72 g 150 µg ad 100 ml	Filter, degas and store at 4°C, pH adjusted by concentrated HCl

Tab. 3-6: Buffers for the HAP (CHT5-I) column

Starting Buffer pH 7.5	10 mM KH_2PO_4 1 mM DTT Water	0.136 g 150 µg ad 100 ml	Filter, degas and store at 4°C
Elution Buffer pH 7.5	500 mM KH_2PO_4 1 mM DTT Water	6.804 g 150 µg ad 100 ml	Filter, degas and store at 4°C

Tab. 3-7: Buffers for HIC (Phenyl sepharose high performance column)

Starting Buffer pH 7.5	50 mM KH_2PO_4 1 M $(NH_4)_2SO_4$ 1 mM DTT Water	0.68 g 13.21 g 150 µg ad 100 ml	Filter, degas and store at 4°C
Elution Buffer pH 7.5	50 mM KH_2PO_4 1 mM DTT Water	0.68 g 150 µg ad 100 ml	Filter, degas and store at 4°C

Tab. 3-8: Buffer for Gel Filtration (HiPrep16/60 Sephacryl S-200 High Resolution column)

Starting and elution Buffer pH 7.5	50 mM Tris 100 mM KCl 1 mM DTT Water	6.057g 7.456 g 1.5 mg ad 1000 ml	Filter, degas and store at 4°C

2.1.5 Buffers and solutions for gel electrophoresis (SDS-PAGE):

Tab. 3-9: Buffers and solutions for gel electrophoresis

Stacking gel	Distilled water 1 M Tris-HCl (pH 6.8) Acrylamide/Bis 30 % 10 % (w/v) SDS 10 % (w/v) APS TEMED	3.4 ml 0.63 ml 0.83 ml 0.05 ml 0.05 ml 5 µl	
Running gel (12 %)	Distilled water 1.5 M Tris-HCl (pH 8.8) Acrylamide/Bis 30 % 10 % (w/v) SDS 10 % (w/v) APS TEMED	3.3 ml 2.5 ml 4.0 ml 0.1 ml 0.1 ml 4 µl	
2x Protein Loading Buffer	Distilled water 0.5 M Tris-HCl (pH 6.8) Glycerine 10 % (w/v) SDS β-Mercaptoethanol 0.5 % (w/v)Bromphenol blue	2.7 ml 1.0 ml 2.0 ml 3.3 ml 0.5 ml 0.5 ml	Stored at 4 °C
10x SDS-Electrode Buffer	Tris base Glycin Na-SDS Water	15 g 72 g 5 g ad 500 ml	Stored at 4 °C, before use 1:10 dilution
Staining Solution	Coomassie-blue R 250 Methanol Acetic acid Water	25 ml 100 ml 20 ml ad 200 ml	
Coomassie-blue	Coomassie-blue R 250 Water	0,5 g 50 ml	Dissolve the dye in water and filtrate
Destaining Solution	Methanol Acetic acid Distilled water	30 ml 20 ml ad 200 ml	
Silver Staining Fixing solution	30% ethanol 10% acetic acid Distilled water	150 ml 50 ml ad 500 ml	Store at room temperature
Sensitizing solution	30 % ethanol 0.5 M sodium acetate 0.2 % $Na_2S_2O_3$ Distilled water	150 ml 20.5 g 1 g ad 500 ml	Store at room temperature
Silver solution	0.2 % silver nitrate 0.01 % formaldehyde Distilled water	0.6 g 30 µl ad 300 ml	Prepare freshly
Developing solution	6 % Na_2CO_3 0.02 % formaldehyde Distilled water	18 g 60 µl ad 300 ml	Prepare freshly
Stop solution	1.5 % Na_2 EDTA Distilled water	4.5 g ad 300 ml	

2.1.6 Buffers for First-Dimension Isoelectric Focusing (IEF):

Tab. 3-10: Buffers for First-Dimension Isoelectric Focusing

Rehydration buffer a	7 M Urea 2 M Thiouea 4 % CHAPS 50 mM DTT Distilled water	4.2 g 1.5 g 0.4 g 0.077 g 10 ml	Shake for 20 min, store in 1 ml aliquots at -80°C
Rehydration buffer b	Rehydration buffer a Ampholyte Triton X-100 Bromophenol Blue (add with pipette tip)	2.5 ml 125 µl 125 µl Trace	Store in 1 ml aliquots at -80°C
SDS-Equilibration buffer	1.5 M Tris-HCL (pH 8.8) Urea SDS Glycerol Bromophenol Blue Distilled water	10 ml 72.07 g 4 g 69 ml Trace ad 200 ml	Store in 50 ml aliquots at -20°C

For second dimension SDS-PAGE (always fresh preparation):

Addition of DTT: add 300 µl of a 1 M DTT solution to 20 ml SDS-equilibration buffer (4 strips) to give a Final concentration of 15 mM.

Addition of iodoacetamide: add 0.56 g to 20 ml SDS-equilibration buffer (4 strips) to give a final concentration of 0.15 M.

2.1.7 Equipment

Balances	Large and small scale	Sartorius
Water bath	Typ 3041	Köttermann
	Exatherm U3	Julabo
Heater block	Dri-Block DB 3D	Techne
Incubator	HT	Infors
Centrifuge	Universal 32R	Hettich
	Biofuge 13	Heraeus Sepatech
HPLC	1525 Binary HPLC Pump	Waters
	2487 Dual λ Absorbance Detector	Waters
	Breeze GBC Software	Waters
FPLC	Biologic-System	Bio Rad
	Controller	Bio Rad
	Fraction collector 2128	Bio Rad
Photometer	Ultraspec 1000	Pharmacia Biotech
Power supply	Standard Power Pack P25	Biometra
	Power Pack 300	Bio Rad
Electrophoresis	Protein-Chamber	Biometra; Bio Rad
Vacuum concentrator	Genelac SF 50	Biometra
pH-Meter	Digital pH Meter 325	WTW (Wissenschaftlich-Technische-Werkstätten)
Autoclave	Vapoklav	Sterilco
Clean bench	LaminAir HLB 2472	Heraeus
Freeze dryer	Alpha	Christ
Water distillation Unit	Milli-Q Reagent Water	Millipore
Magnetic rotator	Vibrax-VXR VF2 Combimag Ret	IKA–Labortechnik (Janke & Kunkel), Staufen
Multiphor™	II Electrophoresis unit	Pharmacia

2.2. Plant material

2.2.1 Cultivation of callus and cell suspension cultures

Callus tissue was grown on solid LS medium (Fig. 2-1). An aliquot was transferred onto new medium every three weeks.

Fig. 2-1: Callus culture of *H. calycinum* on solid LS medium

Callus tissue was suspended in liquid LS medium (Fig. 2-2). The resulting cell suspension cultures were propagated in 200 ml Erlenmeyer flasks at 100 rpm and 25°C in the dark. For growth curves and enzyme assays, 3 g cells were inoculated into 50 ml liquid BDS medium at 7-day intervals (Fig. 2-3).

2.2.2 Cell harvest

Cell cultures were collected by suction filtration through filter paper in a Büchner funnel. The fresh weight was determined.

Fig. 2-2: Cell suspension culture of *H. calycinum* in LS medium

Fig. 2-3: Cell suspension culture of *H. calycinum* in BDS medium

3. Methods

3.1. Syntheses and analytical work

3.1.1 Synthesis of dimethylallyl diphosphate

Dimethylallyl diphosphate was prepared by the method of Cramer and Böhm (1959), modified by Popják et al. (1966). Trichloroacetonitrile (36 mmol) and di-triethylammonium phosphate (14.4 mmol) were added slowly through a dropping funnel to 3,3-dimethylallyl alcohol (6 mmol) at room temperature over a period of 3-4 hours, the mixture being stirred continuously. After standing for another 2 hours at room temperature, the reaction mixture was diluted with 100 ml of ether, transferred to a separating funnel and extracted three times with 25-30 ml of 0.1 N aq. ammonia. The aqueous extracts were combined and concentrated to a few millilitres on a rotary evaporator (bath temperature about 50°C).

3.1.1.1 Purification of synthetic dimethylallyl diphosphate

The aqueous concentrate obtained above was applied to a column of silica gel (0.063-0.200 mm, VWR international company; 60 x 1.3 cm) equilibrated with n-propanol-conc. ammonia (sp. gr. 0.88)-water (6:3:1, by volume). The same solvent system was used for elution. 3 ml fractions were collected. Fractions containing dimethylallyl diphosphate (No. 35-44, eluted after the monophosphate) were combined and concentrated to few millilitres (2-4 ml), made alkaline with a drop of ammonia and freeze dried.

3.1.1.2 Thin layer chromatography (TLC)

For detection of dimethylallyl diphosphate, samples, e.g. aliquots of the above fractions, were spotted onto a TLC plate (silica gel 60 F_{254}, 20 x 20 cm, thickness 0.2 mm; Merck, Darmstadt). The solvent was n-propanol-conc. ammonia (sp. gr. 0.88)-water (6:3:1, by volume). After drying and spraying with ferric chloride-sulfosalicylic acid, phosphate-containing compounds appeared as white spots on a pink background.

3.1.1.3 NMR-analysis

NMR spectra were obtained on a Bruker Advance 400 spectrometer at operation frequencies of 400 MHz (^{1}H) and 101 MHz (^{13}C), respectively.
The solvent was $CDCl_3$ and the chemical shifts were referenced to TMS (± H D 0.00) and to $CDCl_3$ (CD 77.0). The spectrometer software used was XWinNMR version 3.1.

3.1.2 Synthesis of dimethylallylphlorisobutyrophenone

The reaction was conducted according to Li Xiao et al. (1998). 800 mg 2-methyl-1-(2,4,6-trihydroxyphenyl)-propan-1-one (4.1 mmol) was dissolved in 10 ml aqueous KOH (4.9 mmol) and cooled to 0°C. 910 mg prenyl bromide (6.1 mmol) was added dropwise over 5 min at 0°C. After stirring for 1 h at room temperature, the reaction mixture was poured into 10 ml of ice-water, acidified with HCl (10 %) to pH 2 and extracted with ethyl acetate (3 x 10 ml). The combined organic phases were dried over Na_2SO_4 and concentrated under reduced pressure.

3.1.2.1 Purification of synthetic dimethylallylphlorisobutyrophenone

Due to instability in silica gel, the reference compound was chromatographed on Sephadex LH-20 (2.5 x 40 cm; Amersham Biosciences, Freiburg, Germany) using methanol (70 %) as solvent. Further purification was achieved by semi-preparative HPLC (0.8 x 25 cm; Eurosorb-100 C_{18}) using water and methanol as solvents. The flow rate was 3 ml/min and the following gradient was used.

Tab. 3-11: HPLC program

Gradient	Time [min]	Methanol [%]	Water [%]	Wavelength [nm]
	0	50	50	291
	2	50	50	
	32	80	20	
	37	100	0	
	40	100	0	
	42	50	50	
	47	50	50	

3.1.2.2 Thin layer chromatography (TLC)

TLC was carried out on silica gel (3.1.1.2.). The solvent was diethyl ether : pentane (60:40). After drying, detection of dimethylallylphlorisobutyrophenone was under UV-Light.

3.1.2.3 High performance liquid chromatography (HPLC)

3.1.2.3.1 Instrumental

Pumps: Waters 1525 Binary HPLC Pump

Detector: Waters 2487 Dual Absorbance

Software: Waters Breeze GPC

Column: C_{18} 100-5 (25 x 0.4 cm; Macherey-Nagel, Düren, Germany)

HPLC photo diode array detection

Injector: Waters712 auto sampler

Detector: Waters 991 photo diode array

Pumps: Waters

Controller: Waters 600E system

Column: C_{18} 100-5 (25 x 0.4 cm; Macherey-Nagel, Düren, Germany)

3.1.2.3.2 Mobile phases

Enzymatic products were analyzed by HPLC using linear gradients of methanol (solvent A) and bidistilled water (solvent B), both containing 1% (v/v) *ortho*-phosphoric acid (Tab. 3-12)

Tab. 3-12: HPLC programs

Gradient	Time [min]	Methanol [%]	Water [%]	Wave length [nm]
1	0	50	50	291
	2	50	50	
	32	80	20	
	37	100	0	
	40	100	0	
	42	50	50	
	47	50	50	
2	0	50	50	291
	5	50	50	
	20	100	0	
	25	100	0	
	27	50	50	
	3	50	50	

3.1.2.4 GC-MS – Analysis

3.1.2.4.1 Instrumental

Precolumn: 2 m x 0.32 mm fused silica (Phenomenex)
Analytical column: 30 m x 0.32 mm ZB1 (Phenomenex)
Injection volume: 1µl
Injector and transfer line: 280 °C
Temperature program: 100 °C (3 min) - 300 °C (5 min) at 10 °C min^{-1}
Split ratio: 1:20
Gas flow: 1.6 ml/min He

The capillary column was directly coupled to a triple quadruple mass spectrometer Finnigan TSQ 700. The retention index (RI) was calculated by a set of hydrocarbons (even numbered C_{12} to C_{28}) by linear interpolation.

3.1.2.4.2 Sample preparation

The synthetic reference compounds (dimethylallylphlorisobutyrophenone and dimethylallylphloracetophenone) and enzymatic products were first acetylated. The products were dissolved in 10µl dry pyridine and 10 µl acetic acid anhydride. After 1 h at room temperature, the reaction was quenched by addition of 100 µl methanol, and an aliquot was analyzed by GC-MS.

3.1.3 Synthesis of dimethylallylphloracetophenone

The reaction was conducted according to Li Xiao et al. (1998). 2,4,6-Trihydroxyacetophenone (7 mmol) was dissolved in 5.3 ml aq. KOH (10%) and cooled to 0 °C. Prenyl bromide (10.5 mmol) was added dropwise over 5 min. After stirring for 1 h at room temperature, the reaction mixture was poured into 10 ml of ice-water, acidified with HCl (10 %) to pH 2 and extracted with ethyl acetate (3 x 10 ml). The combined organic phases were dried over Na_2SO_4, and concentrated under reduced pressure.

3.1.3.1 Purification of synthetic dimethylallylphloracetophenone

The concentrate obtained in the previous step was chromatographed on silica gel (0.063-0.200 mm, VWR international company; 60 x 1.3 cm) using different solvent mixtures (A: ethyl acetate : dichloromethane : acetic acid (10.5 : 80.5 : one drop); B: diethyl ether : pentane (60 : 40)). The fractions containing the desired compound were combined and concentrated on a rotary evaporator.

3.1.3.2 Thin layer chromatography (TLC)

TLC was carried out on silica gel (3.1.1.2) using the above solvent (3.1.3.1). Detection was under UV-light.

3.2. Enzyme extraction and incubation

3.2.1 Preparation of cell-free extracts

Cultured cells (5g) were mixed with 0.5 g Polyclar AT (to adsorb phenolics) and a spatula of seasand. The mixture was homogenised for 15 min in 5 ml of 0.1 M KH_2PO_4 buffer, pH 6.5 containing 10 mM DTT (to protect SH groups). After centrifugation at 13000 rpm at 4 °C for 15 min, the supernatant was passed through a PD10 column and the high-molecular weight fraction was eluted with 3.5 ml of 0.1 M Tris-HCl buffer (pH 7.5).

3.2.2 Protein determination

Protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. To obtain a calibration curve, aliquots containing 1 µg-10 µg BSA was mixed with 900 µl freshly prepared Bradford dye solution. The volume was made up to 1 ml with water and the contents in each cuvett were mixed carefully. After 5 min the absorbance of the samples at 595 nm was measured using a blank of 900 µl Bradford solution + 100 µl water. The same procedure was applied to aliquots of crude and partially

purified enzyme extracts, and protein concentrations were determined from the calibration curve.

3.2.3 Enzyme assay

The standard prenyltransferase assay was carried out in a final volume of 250 µl containing 1 mM 2,4,6 trihydroxyisobutyrophenone, 2 mM DMAPP, 10 mM $FeCl_2$ and 0.1 M Tris-HCl buffer (pH 7.5). The reaction was initiated by addition of an aliquot of enzyme preparation. After incubation for 30 min at 37°C, the products were extracted twice with 0.5 ml ethyl acetate by vigorous mixing for 1 min and centrifugation at 13.000 rpm for 5 min. The organic phase was evaporated to dryness and the residue analyzed by HPLC.

3.2.4 Enzyme preparation for ultracentrifugation

For the preparation of microsomes, 6 ml crude enzyme extract (0.9 mg protein /ml) were centrifuged at 100 000 g for 30 min. The pellet was resuspended in 2.5 ml phosphate buffer (0.1 M, pH 6.5) containing 1 mM DTT.

3.2.5 Linearity with protein amount and incubation time

The amount of enzymatically formed dimethylallylphlorisobutyrophenone was determined as a function of the protein amount in the standard assay (20 µg, 40 µg, 80 µg, 100 µg, 150 µg) and the incubation time (0.5, 10, 20, 30, 40, 50, 60 min). All incubations were performed in duplicate and average values were calculated.

3.3. Characterization of the prenyltransferase

3.3.1 Determination of pH and temperature optima

Enzyme assays were carried out at pH values from 5.0 to 11.0 using two buffers for optimum buffer capacity, 0.1 M phosphate buffer (5-7) and 0.1 M Tris-HCL buffer (7.5- 11). At the optimum pH value, another series of incubations were performed at different temperatures between 15 to 55 °C.

3.3.2 Study of substrate specificity

At the pH and temperature optima, enzyme assays were performed using a series of prenyl donors and prenyl acceptors. All substrate concentrations were saturating.

3.3.3 Cofactor requirement

The divalent metal ions (Fe^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}) were tested as their chloride salts at concentrations of 10 mM in the presence and absence of 10 mM ascorbic acid as reducing agent.

3.3.4 Determination of K_m values

The kinetic properties were calculated from Lineweaver-Burk plots. For DMAPP, the concentration was changed between 0.2 and 16 mM while that of phlorisobutyrophenone was kept constant at 2 mM. For phlorisobutyrophenone, the concentration varied between 0.1 and 8 mM while the assays contained 2 mM DMAPP. For ferrous, the concentration was changed between 1 and 20 mM while the assays contained 2 mM DMAPP and 2 mM phlorisobutyrophenone. Two independent experiments were performed and mean values calculated.

3.4. Purification of the prenyltransferase

All steps of the purification were carried out at 0-4°C (on ice, in the refrigerator). Before samples were loaded on a column they were filtered through an unsterile membrane filter (25 mm, 0.45 µm pore diameter; Roth, Karlsruhe) to avoid clogging of FPLC columns.

3.4.1.1 Ammonium sulphate precipitation

Cell-free extract (50 ml) obtained from 100 g of freshly harvested cells was fractionated by addition of ammonium sulphate in 10 % steps up to 70 % saturation. The enzyme extract was mixed with fine powder of ammonium sulphate under continuous stirring at 4°C for 30 min and centrifuged at 9000 rpm for 35 min. The protein pellet was dissolved in 2.5 ml phosphate buffer pH 7.5 and desalted by passing through a PD10 column equilibrated with Tris-HCl buffer pH 7.5. This buffer (3.5 ml) was also used to elute the high molecular mass fraction. The protein fraction precipitating between 30-65 % ammonium sulphate saturation was used for the next purification step.

3.4.1.2 DEAE-anion exchange chromatography

3.4.1.2.1 DEAE-column with 1 ml gel volume

Cultured cells (20 g) were homogenized in 10 ml extraction buffer (2.1.3) and the crude extract (17 ml) was fractionated by addition of ammonium sulphate. The protein fraction which precipitated between 30-65 % saturation (3.4.1.1) was dissolved in 2.5 ml extraction buffer and desalted by passing through a PD10 column equilibrated with DEAE starting buffer (2.1.4). The same buffer (3.5 ml) eluted the protein fraction which was applied to a Sepharose™ Fast Flow DEAE column (weak anion) with 1 ml gel volume (Amersham Bioscience). Unbound proteins were removed by washing with three bed volumes of DEAE starting buffer and bound proteins were eluted with a linear gradient from 0-0.5 M KCl, i.e increasing volumes of DEAE elution buffer (2.1.4). The total gradient volume was 20 ml and the flow rate 2ml/min. Fractions of 1 ml were collected. The FPLC program for this chromatographic separation is given in Table 3-13.

Tab. 3-13: FPLC program for a 1ml-DEAE- anion exchange column

Step	Time[min]	Function	Buffer	Volume /Flow
1	13.0	collect fractions within 1 time window ending at 33 ml		
2	0.0	UV Lamp	ON	
3	0.0	Set zero baseline	UV detector	
4	0.0	isocratic flow	Start buffer 100 % Elution buffer 0 %	vol. 4.0 ml flow 2 ml/min
5	4.0	load/inject sample	load sample static loop auto inject valve	vol. 3.0 ml 0.5 ml/min
6	7.0	isocratic flow	Start buffer 100 % Elution buffer 0 %	vol. 6.0 ml 2 ml/min
7	13.0	linear gradient	Start buffer 100 %→0 % Elution buffer 0 %→100 %	vol. 20.0 ml 2 ml/min
8	33.0	isocratic flow	Start buffer 0 % Elution buffer 100 %	vol. 4.0 ml 2 ml/min
9	37.0	isocratic flow	Start buffer 100 % Elution buffer 0 %	vol. 6.0 ml 2 ml/min
10	37.0	End of protocol		
11	37.0	UV Lamp	OFF	

3.4.1.2.2 DEAE-column with 5 ml gel

The procedure described under (3.4.1.2.1) was used, except that 60 g cells and a 5 ml column were employed. Furthermore, the volume of the total gradient was 50 ml and the flow rate 2ml/min. Fractions of 3 ml were collected. The FPLC program for this chromatographic separation is given in Table 3-14.

Tab. 3-14: FPLC program for a 5ml-DEAE- anion exchange column

Step	Time[min]	Function	Buffer	Volume /Flow
1	40.0	collect fractions within 1 time window ending at 90 ml		
2	0.0	UV Lamp	ON	
3	0.0	set zero baseline	UV detector	
4	0.0	isocratic flow	Start buffer 100 % Elution buffer 0 %	vol. 15.0 ml flow 2 ml/min
5	15.0	load/inject sample	load sample static loop auto inject valve	vol. 5 ml 0.5 ml/min
6	20.0	isocratic flow	Start buffer 100 % Elution buffer 0 %	vol. 20.0 ml 2 ml/min
7	40.0	linear gradient	Start buffer 100 %→0 % Elution buffer 0 %→100 %	vol. 50.0 ml 2 ml/min
8	90.0	isocratic flow	Start buffer 0 % Elution buffer 100 %	vol. 15.0 ml 2 ml/min
9	105.0	isocratic flow	Start buffer 100 % Elution buffer 0 %	vol. 15.0 ml 2 ml/min
10	120.0	End of protocol		
11	120.0	UV Lamp	OFF	

3.4.1.3 Hydroxylapatite adsorption chromatography

Cells (60 g) were extracted with 30 ml extraction buffer (2.1.3) and the protein extract (17 ml) was fractionated by addition of ammonium sulphate. The protein fraction which precipitated between 30-65 % ammonium sulphate saturation (3.4.1.1) was dissolved in 7.5 ml extraction buffer and desalted by passing through a PD10 column equilibrated with HAP starting buffer (2.1.4). The high molecular mass fraction was eluted with 3.5 ml of the same buffer and applied to a Bio-Scale Ceramic Hydroxyapatite®-Column type I (10 x 64 mm, Bio-Rad). Unbound proteins were removed by washing with three bed volumes of HAP starting buffer and bound proteins were then eluted with a linear gradient from 10-500 mM phosphate by increasing the percentage of HAP elution buffer (2.1.4). The total volume of the gradient was 50 ml at a flow rate of 2 ml/min. Fractions of 3 ml were collected.

3.4.1.4 Hydrophobic interaction chromatography

Protein extraction and ammonium sulphate fractionation were as described under (3.4.1.2.1). The protein eluted from the PD10 column in HIC starting buffer (2.1.4) was applied to a Phenyl Sepharose® HP-column with 1 ml gel volume (Amersham Bioscience) also equilibrated with HIC starting buffer. Unbound proteins were removed by washing with three bed volumes of the same buffer and bound proteins were eluted with a linear gradient from 1 M $(NH_4)_2SO_4$ (100 % HIC start buffer) to 0 M $(NH_4)_2SO_4$ (100 % HIC elution buffer). The gradient volume was 20 ml at a flow rate of 2 ml/min. Fractions of 1 ml were collected.

3.4.1.5 Mono-Q anion exchange chromatography

Cultured cells (15 g) were extracted with 7.5 ml extraction buffer (2.1.3) and the enzyme extract (14 ml) was fractionated by addition of ammonium sulphate. The protein pellet obtained between 30-65 % saturation (3.4.1.1) was dissolved in 2.5 ml extraction buffer and desalted by passing through a PD10 column equilibrated with Mono-Q starting buffer (2.1.4). The same buffer (3.5 ml) served to elute the protein fraction which was applied to a Sepharose™ Fast Flow Mono-Q column (strong anion) with 1ml gel volume (Amersham Bioscience). Unbound proteins were desorbed by washing with three bed volumes of Mono-Q starting buffer and bound proteins were eluted with a linear gradient from 0-0.5 M KCl by increasing the percentage of Mono-Q elution buffer (2.1.4). The gradient volume was 20 ml at a flow rate of 2 ml/min. Fractions of 1 ml were collected.

3.4.1.6 Gel filtration

For molecular mass estimation and as one step in the purification protocol, a HiPrep16/60 Sephacryl S-200 High Resolution column was used. Determination of the molecular mass requires preceding calibration of the column by using standard proteins :

- Albumin, Bovine serum, 66 kDa
- Alcohol dehydrogenase, Yeast, 150 kDa
- β-Amylase, Sweet Potato, 200 kDa
- Blue dextran, 2000 kDa
- Carbonic anhydrase, Bovine erythrocytes, 29 kDa
- Cytochrome c, Horse heart, 12.4 kDa

The column was equilibrated with 50 mM Tris –HCl, pH 7.5 containing 100 mM KCl. To determine the void volume (V_o), blue dextran (2000 kDa) was dissolved in equilibration buffer (2 mg/ml) and passed through the gel filtration column at a flow rate of 0.3 ml/min. The fraction volume was 5 ml. To determine the elution volume (V_e) of the standards, the proteins were dissolved in equilibration buffer at the following concentrations (Tab. 3-15).

Tab. 3-15: Recommended concentrations of standard proteins

Standard protein	Concentration used
Albumin	10 mg/ml
Alcohol dehydrogenase	5 mg/ml
β-Amylase	4 mg/ml
Carbonic anhydrase	3 mg/ml
Cytochrome c	2 mg/ml

The individual samples (2 ml) were applied to the column. A standard curve was calculated by plotting the molecular mass vs. V_e/V_o for each standard protein.

To estimate the relative molecular mass of prenyltransferase, a crude extract from 20 g cells was subjected to ammonium sulphate precipitation and DEAE chromatography. The fractions with highest prenyltransferase activity were combined and applied to the gel filtration column under the above conditions for blue dextran and protein standards. After measuring which fractions contain the enzyme activity, the elution volume (V_e) of the prenyltransferase and the V_e/V_o value were determined. The relative molecular mass was concluded from the standard curve.

3.4.2 Purification of the prenyltransferase to apparent homogeneity

A large batch of cell suspension cultures (1379 g) was grown in BDS medium (2.2.1) harvested (2.2.2) and extracted (3.2.1). The chromatographic steps described above were combined in the following order :

1) Ammonium sulphate precipitation
2) DEAE-anion exchange chromatography (column with 5 ml gel volume)
3) Hydroxylapatite-adsorption chromatography (column with 5 ml gel volume)
4) Hydrophobic interaction chromatography (column with 1 ml gel volume)
5) Mono-Q anion exchange chromatography (column with 1 ml gel volume)
6) Gel filtration

Active fractions were localized by measuring prenyltransferase activity, pooled and passed through PD10 columns equilibrated with the starting buffer for the next column chromatography.

3.5. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is used to determine the grade of purity and the subunit molecular mass of proteins. These were denatured by heating in the presence of the anionic detergent SDS and SH reagent to reduce S-S bonds. Thereby, the proteins are uniformly negatively charged and disrupted into subunits, respectively. While migrating towards the anode, they are separated according to their molecular mass (Laemmli, 1970).

3.5.1 Preparation of gel and samples

Polyacrylamide gels are formed by cross-linking the acrylamide monomer with N´-methylene bisacrylamide promoted by the addition of ammoniumpersulfate (APS) and TEMED (*N, N, N´, N´*-Tetramethylethylenediamine). TEMED also acts as stabilizing agent. First, the applied protein mixture is concentrated in a highly porous stacking gel and then

separated in the resolving gel. The concentration of acrylamide and bisacrylamide in the resolving gel was 12% (2.1.4) which allowed high resolution of proteins between 70 and 12 kDa. Immediately after the addition of APS and TEMED, the mixture for the resolving gel was poured between two vertically oriented glass plates in a gel-casting stand to make a gel of 0.75 mm thickness. Protein samples to be analysed were mixed with protein loading buffer (ratio 1:2) and denatured at 95°C for 5 min, followed by loading the samples into the wells of the stacking gel. The maximum capacity of the wells depended on the tooth number of the comb used to cast the gel, e.g. 15 µl for a 24 –tooth comb.

3.5.2 Electrophoresis and detection of the protein bands

Electrophoresis was carried out on a Minigel –Twin Electrophoresis system (Biometra). The gel was run in 1x SDS electrode buffer at 200 V, 75 mA and 15 W supplied by a Multidrive XL-power supply (Pharmacia) for approximately 90 min.

The protein bands were visualized by two methods:

1- Coomassie Blue staining:

Incubate the gel in Coomassie Blue staining solution (1.2.4) for 60 min or overnight, followed by destaining for 60 min in a destaining solution (1.2.4).

2- Silver staining:

Process the gel according to the steps given in (Table 2-16). Use 250 ml of each solution (1.2.4) per gel. The timing of the steps differs according to the type of solution and is indicated in the table. Shake slowly on a shaker or rocker

Tab. 3-16: **Silver staining protocol**

Solution	Time for gel incubation
Fixing solution	40-60 min
Sensitizing solution	60 min or over night
Distilled water	3 x 5 min
Silver solution	30 min
Distilled water	2 x 2 min
Developing solution	Until protein bands are visible (5-10 min)
Stop solution	10 min

3.6. Two-dimensional (2-D) electrophoresis

2-D electrophoresis is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. The technique sorts proteins according to two independent properties in two discrete steps: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI). The second-dimension step, SDS-PAGE, separates proteins according to their subunit molecular masses (M_r). Each spot on the resulting two-dimensional array corresponds to a single protein species in the sample. The power of 2-D electrophoresis as a biochemical separation technique has been improved to achieve rapid analysis of proteins. Single spots eluted or transferred from 2-D gels can be rapidly identified. Mass spectrometric techniques have been developed that allow analysis of very small quantities of peptides and

proteins. Chemical microsequencing and amino acid analysis can be performed on increasingly smaller samples. Immunochemical identification is now possible with a wide assortment of available antibodies.

3.6.1 Sample preparation

The protein fraction to be loaded on a 2-D gel must be present in a low ionic strength denaturing buffer that maintains the native charges of proteins and keeps them soluble. Salt is the major cause of poor focusing in isoelectric focusing (IEF) and increases focusing time, as proteins will migrate to their pI only after the ions have moved out of the strips. The salt is easily removed by protein precipitation using TCA.

TCA precipitation protocol:

1) Mix an aliquot of the purified protein (500 µl) with trichloroacetic acid (TCA) to give a 13% final concentration. Keep 5 min at -20°C and then 15 min at 4°C.

2) Spin 15 min at 4°C in a microcentrifuge at maximum speed (15000 g). Carefully discharge the supernatant and retain the pellet.

3) Wash the pellet with 200 µl cold acetone, spin 5 min at 15000 g.

4) Repeat step 3 twice, dry tube by inversion on tissue paper.

3.6.2 First–dimension isoelectric focusing (IEF)

1. Resuspend the pellet in 85 µl rehydration buffer a (2.1.6.).

2. Add 45 µl rehydration buffer b (2.1.6.) and mix.

3. Pipet the indicated volume (125 µl) of the sample as a line along the back edge of a channel

in rehydration tray. The line of sample should extend along the whole length of the channel except for 1 cm at each end. Avoid to introduce any bubbles which may interfere with the even distribution of the sample in the strip.

4. Peel the coversheet from ReadyStrip IPGstrip (pH 3-10) using forceps, gently place it, gel side down, onto the sample. The "+" and the pH range marked on the strip should be legible and also take care not to trap air bubbles beneath the strip. If this happens, carefully use the forceps to lift the strip up and down from one end until the air bubbles move to the end and out from under the strip.

5. Wait for approximately 1 hr after starting rehydration, until most of the liquid has been absorbed by the strip. Then overlay the strip with 2-3 ml mineral oil to prevent evaporation during the rehydration process.

6. Cover the rehydration tray with the plastic lid provided and leave the tray sitting on a level bench overnight (11-16 h) to rehydrate the IPG strip and load the protein sample.

7. Place a clean, Multiphor™ II Electrophoresis unit onto the lab bench, using forceps, place wick at both ends of wire eclectrodes, pipet 8 µl of bidistilled water onto each wick to wet them.

8. Remove the IPG strip from rehydration tray using forceps, carefully hold the strip vertically for about 7-8 sec. And blot the tip of the strip on a piece of filter paper to allow the mineral oil to drain, then transfer the IPG strip to the cooling plate in the Multiphor™ II, maintain the gel side down and the "+" marked on the strip should be positioned at the side of the plate "+" marked

9. Cover the IPG strip with 2-3 ml fresh mineral oil. Try to avoid air bubbles.

10. Place the lid on the Multiphor II unit and connect the leads on the lid to the power supply and begin focusing program (Tab. 3-17) at 20 °C, 2 steps.

Tab. 3-17: Focusing program

Step	Voltage (V)	Current (mA)	Power (W)	Duration (h:min)	Duration (vh)
1	250	00.1	0.3	1:30	150
2	300	00.1	0.3	0:30	9000

3.6.3 Second-dimension SDS-PAGE

Prior to running the second dimension, it is necessary to equilibrate the IPG strip in a buffer that contains SDS.

1. Remove the mineral oil by placing the IPG strip (gel side up) onto a piece of dry filter paper and blotting with a second piece of wet filter paper.

2. Transfer the blotted IPG strip (gel side up) to the equilibration tray and add 5 ml SDS-equilibtion buffer containing DTT (2.1.6.).

3. Place the tray on a shaker and gently shack for 15 min.

4. Discard the SDS equilibration buffer with DTT from the tray and add 5 ml SDS equilibration buffer containing iodoacetamide (2.1.6).

5. Return the tray to the shaker for 15 min.

6. Discard the SDS equilibration buffer with iodoacetamide from the tray by decanting at the end of the incubation period.

7. Finish preparing the SDS-PAGE gel by blotting away any excess of water remaining inside the IPG well using blotting paper.

8. Remove an IPG strip from the equilibration tray and dip briefly into the graduated cylinder containing 1x SDS electrode buffer (1.2.5.).

9. Lay the IPG strip onto the back of the SDS-PAGE gel above the IPG well, using a spatula to push carefully the IPG strip into the well, try to avoid air bubbles.

10. To ensure good contact between the gel and the IPG strip, overlay it with 0.5-1.0 % molten agarose solution prepared in 1x SDS electrode buffer (1.2.5.). A small amount of Bromophenol Blue can be added to the agarose overlay in order to track the ion front during the run.

11. Load the gel into the electrophoresis cell and fill the reservoirs with 1x SDS electrode buffer (1.2.5.) and begin the electrophoresis, at constantly 200 V. The approximate running time is 35 min.

3.6.4 Detection and analysis of 2-D protein spots

Protein spots were visualized by staining with Coomassie Blue (3.5.2.). The 2-D gel was placed on a light box and the protein spots were cut off with a spatula. The gel pieces were transferred into eppendorf tubes and washed with distilled water 5 times. After drying in a speed vacuum concentrator, the gel pieces were preserved in a freezer at –20 ºC. Tryptic digest and partial sequencing were kindly performed by Dr. Nimtz, GBF (Gesellschaft für Biotechnologische Forschung), Braunschweig.

4. Results

4.1. Detection of prenyltransferase activity

Cell–free extracts from 3-day-old *H. calycinum* cell cultures (3.2.1; 3.2.2) were incubated with phlorisobutyrophenone and DMAPP in the presence of $FeCl_2$ (3.2.3). These incubations resulted in the formation of an enzymatic product, as demonstrated by HPLC (Fig. 4-1) and GC-MS analysis (Fig. 4-2). No enzymatic product was detected when boiled protein extract was used and either phlorisobutyrophenone or DMAPP was omitted.

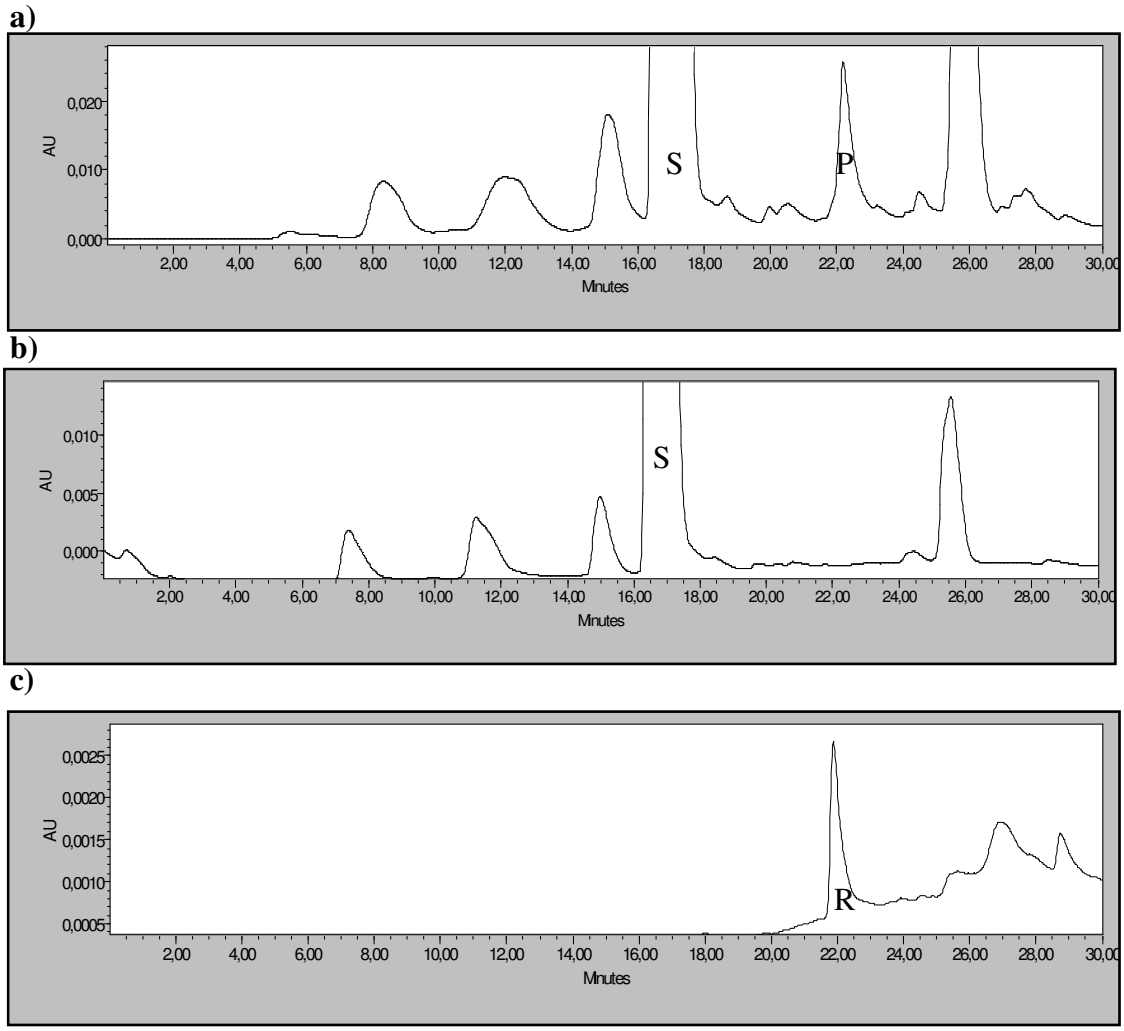


Fig. 4-1: HPLC analysis of prenyltransferase assays with phlorisobutyrophenone. Detection wavelength 291 nm

a) Standard assay

b) Incubation with denatured protein

c) Reference compound dimethylallylphlorisobutyrophenone

P = Enzymatic product (dimethylallylphlorisobutyrophenone)
S = Substrate (phlorisobutyrophenone)
R = Reference compound

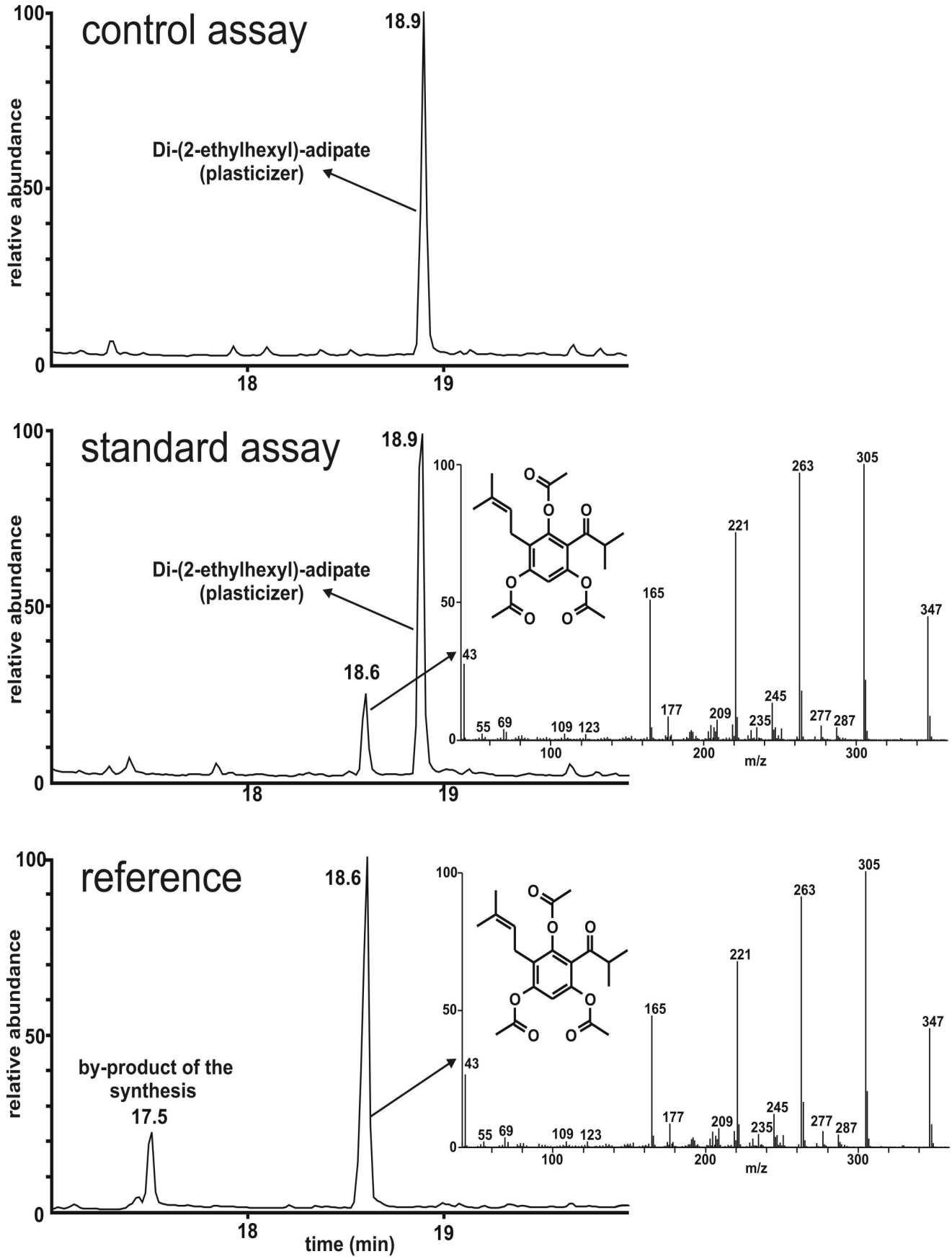


Fig. 4-2: GC-MS analysis of prenyltransferase assays. The control assay contained heat-denatured protein. The enzymatic product and the synthetic reference compound were acetylated.

4.1.1 Synthesis of the reference compound dimethylallylphlorisobutyrophenone

The hyperforin skeleton is phlorisobutyrophenone which is stepwise prenylated. The expected product of the first prenylation step is dimethylallylphlorisobutyrophenone (3-(3-methyl-2-butenyl)-2,4,6-trihydroxyisobutyrophenone) [$C_{15}H_{20}O_4$]. This compound was not commercially available as reference compound and thus had to be synthesized chemically.
The synthesis and the purification of the reaction product are described under (3.1.2) and (3.1.2.1), respectively. The purified product showed an absorption maximum at 291 nm (Fig. 4-3). Its molar mass was 264.07 g/mol (Fig. 4-4).

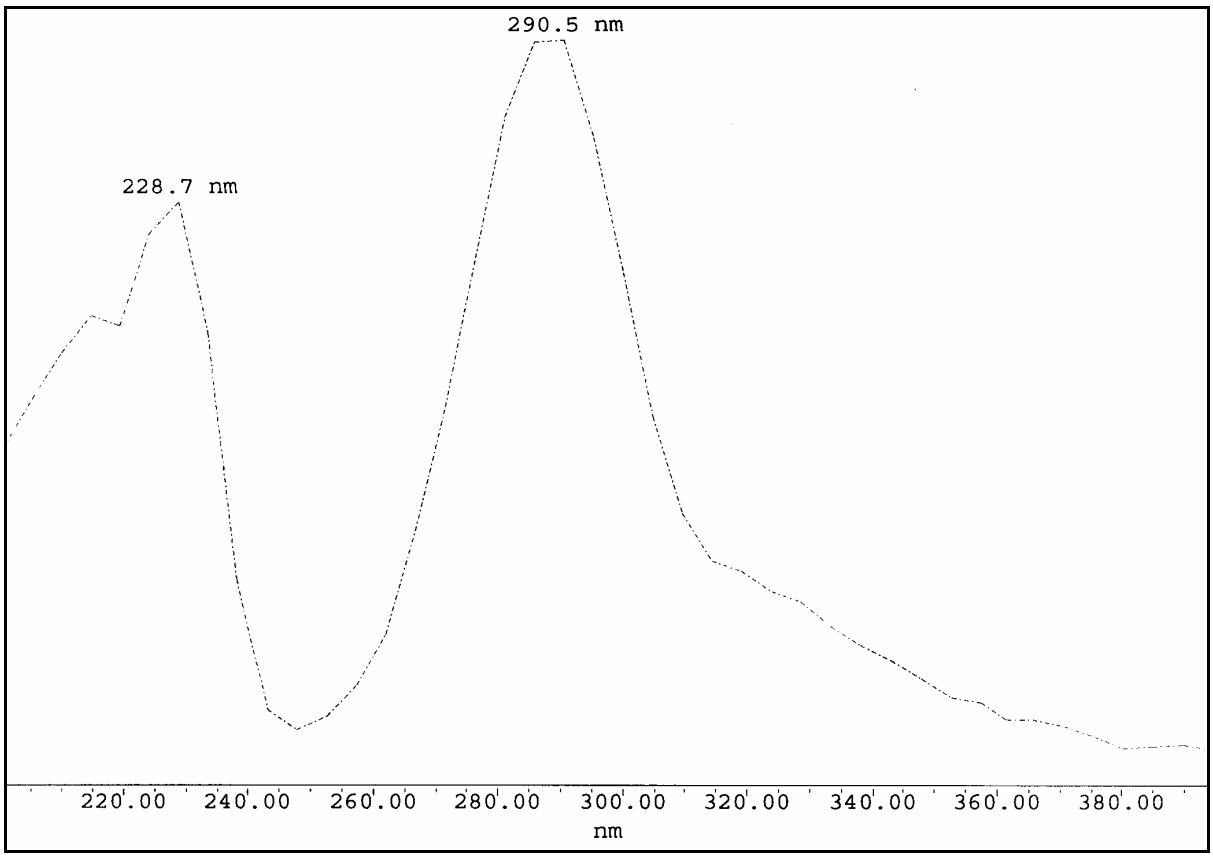


Fig. 4-3: UV spectrum of the synthetic reference dimethylallylphlorisobutyrophenone

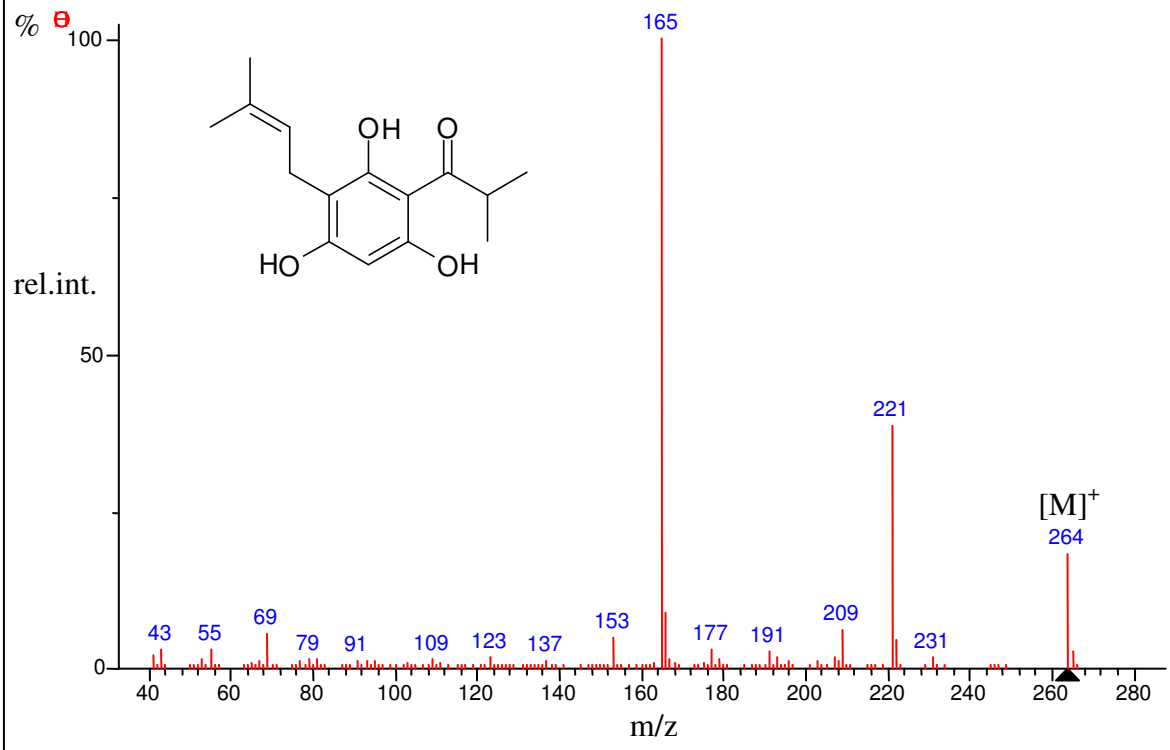


Fig. 4-4: Mass spectrum of the synthetic reference dimethylallylphlorisobutyrophenone

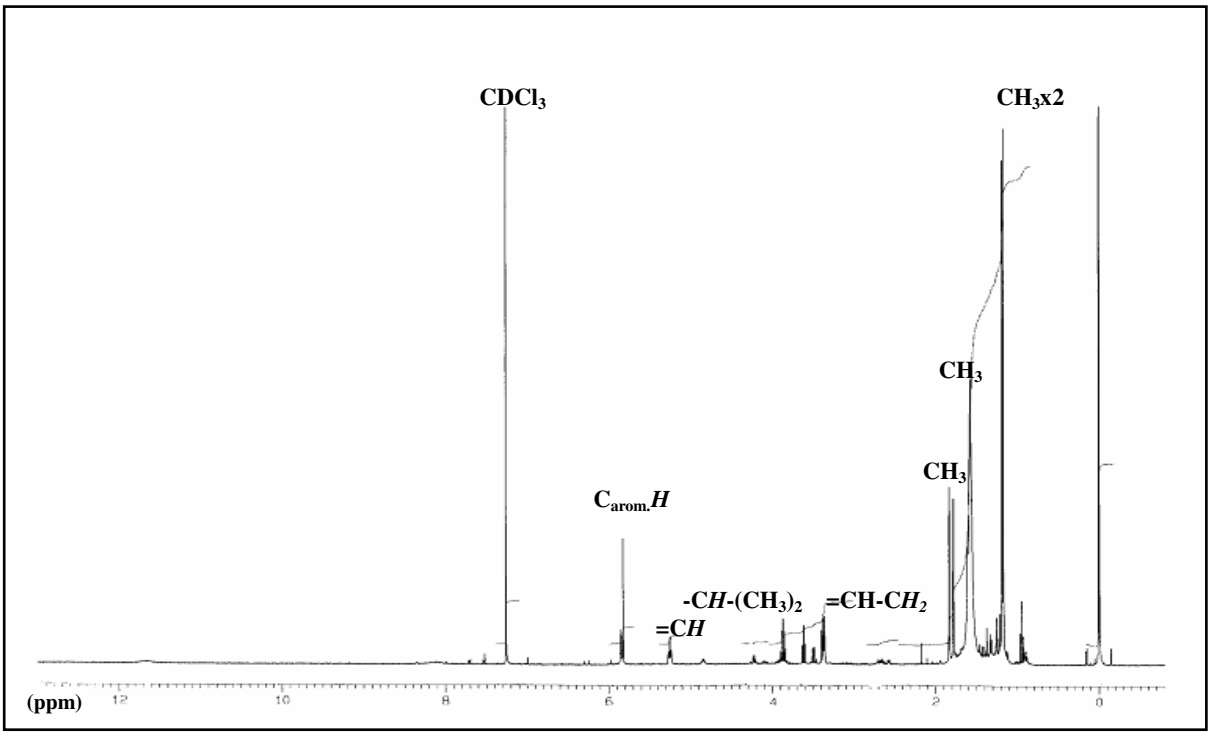


Fig. 4-5: ^{1}H NMR spectrum of the synthetic reference dimethylallylphlorisobutyrophenone

The ^{1}H NMR spectrum is depicted in Fig. 4-5. In detail, the properties were as follows: R_f. (silica gel, diethyl ether : pentane 60 : 40) 0.75; RI (ZB1 / ZB5) 2223 / 2274; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.18 (6H, d, J=7 Hz, CH_3x2), 1.78 (3 H, d, J=1 Hz, CH_3), 1.82 (3 H, s, CH_3), 3.36 (2 H, d, J=7.2 Hz, =CH-C*H*$_2$), 3.8 (1H, qq, -C*H*-(CH_3)$_2$), 5.25 (1H, m, =C*H*) 5.8 (1 H, s, $C_{arom.}$*H*); EIMS, 70eV, m/z (rel. int.): 264 (18, $[M]^+$), 221 (39), 209 (6), 191 (3), 177 (3), 165 (100), 153 (5), 69 (5), 55(3), 43 (3).

These spectroscopic properties of 3-(3-methyl-2-butenyl)-2,4,6-trihydroxyisobutyrophenone agreed with the literature (Kuhnke and Bohlmann, 1985; Fung et al., 1994).

4.1.2 Analysis of the enzymatic product dimethylallylphlorisobutyrophenone

The enzymatic product was extracted from large-scale incubations, purified by TLC (3.1.2.2) and analyzed by GC-MS (3.1.2.4).
In comparison with the sample of chemically synthesized reference compound, it was identified as dimethylallylphlorisobutyrophenone. The synthetic compound and the enzymatic product exhibited identical retention times and mass spectra. This was also true for their acetylated derivatives (Fig. 4-1).

4.2. Characterization of the prenyltransferase

4.2.1 Linearity with protein amount and incubation time

The prenyltransferase reaction was linear with a protein amount up to 80 µg in the assay (Table 4-1) and with time up to 30 min (Fig. 4-6). Product amounts were determined by HPLC using a standard solution of the synthetic reference compound.

Table 4-1: Effect of the amount of protein in the standard assay on the amount of enzyme product.

Amount of protein (µg)	Amount of product (ng)
20 µg	56 ng
40 µg	65 ng
80 µg	108.7 ng
100 µg	182.6 ng
150 µg	106.6 ng

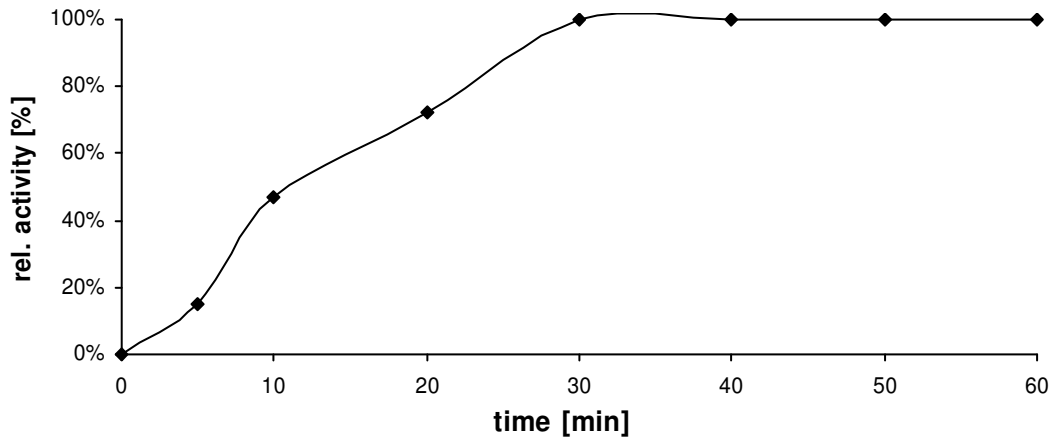


Fig. 4-6: Time course of product formation in the prenyltransferase assay.

4.2.2 Determination of pH and temperature optima

The enzyme showed a broad pH optimum from 6.5 to 9 (Fig. 4-7), and a temperature optimum around 38 °C (Fig. 4-8). An incubation temperature of 37 °C and pH 7.5 were used for the following experiments of enzyme characterization.

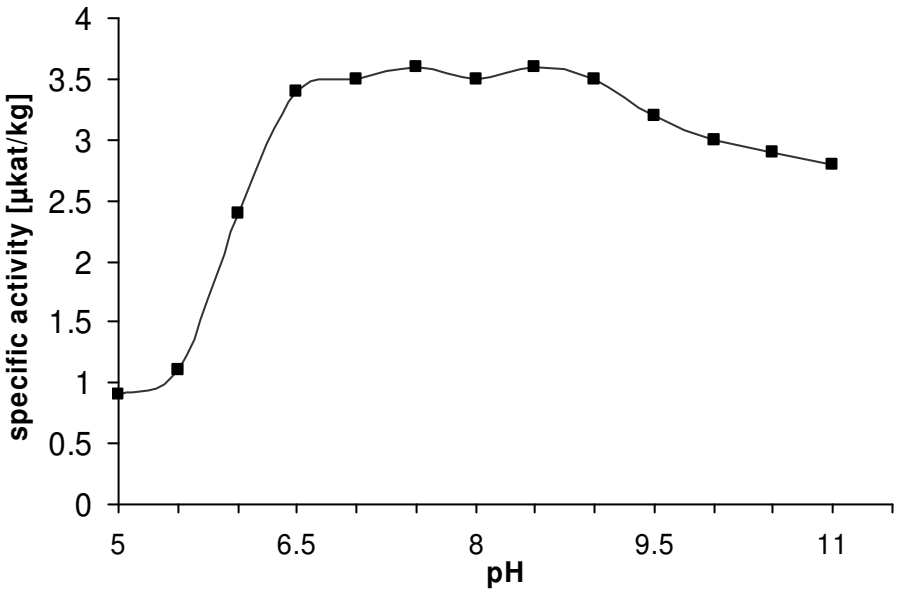


Fig. 4-7: pH optimum of prenyltransferase.

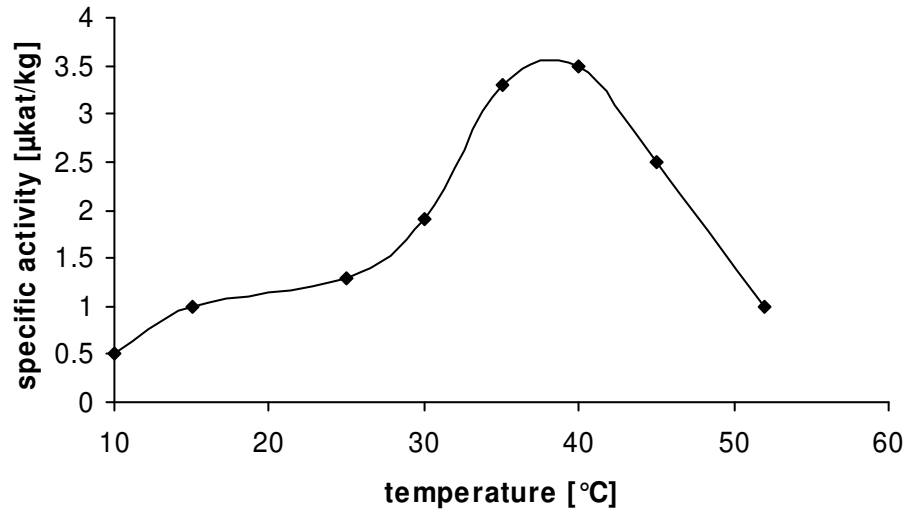
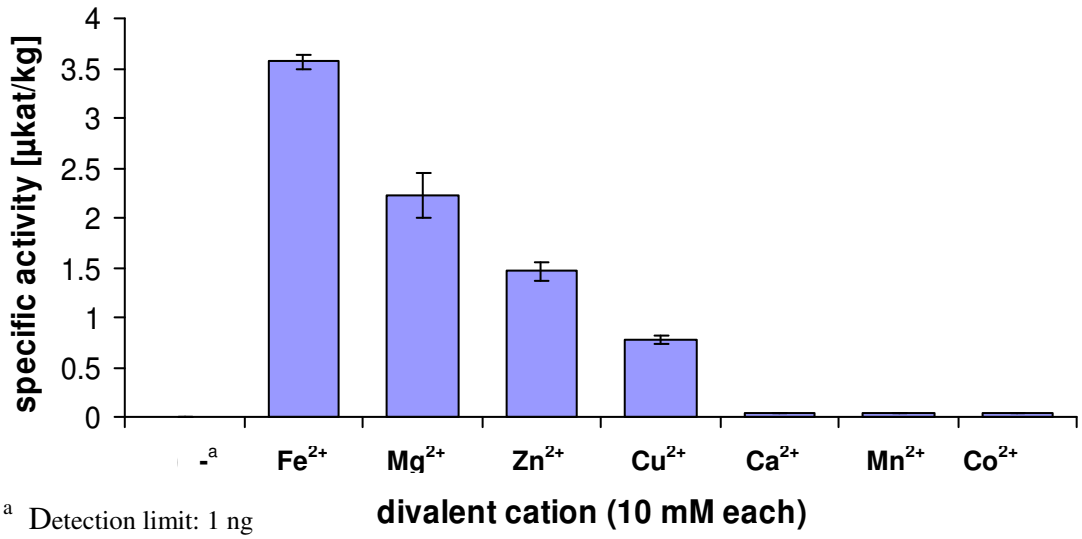


Fig. 4-8: Temperature optimum of prenyltransferase.

4.2.3 Cofactor requirement

Prenyltransferase activity was strictly dependent on a divalent cation, with Fe^{2+} being the most efficient cofactor (Fig.4-9). Lower activities were observed with Mg^{2+}, Zn^{2+}, and Cu^{2+}. No activity was found in the absence of a divalent cation. Prenyltransferase activity increased with increasing concentration of Fe^{2+} (Fig. 4-10). Concentrations higher than 10 mM, however, had an inhibitory effect on the enzyme activity.

The K_m value was 3.8 mM, as calculated from a Lineweaver-Burk plot (Fig. 4-11). Because of its inhibitory effect, the 20 mM concentration was omitted from the double-reciprocal plot.



[a] Detection limit: 1 ng

Fig. 4-9: Effect of divalent cations on dimethylallyltransferase activity. Bars represent means and standard deviations from three independent determinations.

4.2.4 Effect of ascorbic acid

To find out whether or not the preference of the enzyme for ferrous was due to the reducing properties of this ion, assays in the presence and the absence of 10 mM ascorbic acid were carried out. Both experiments gave the same result.

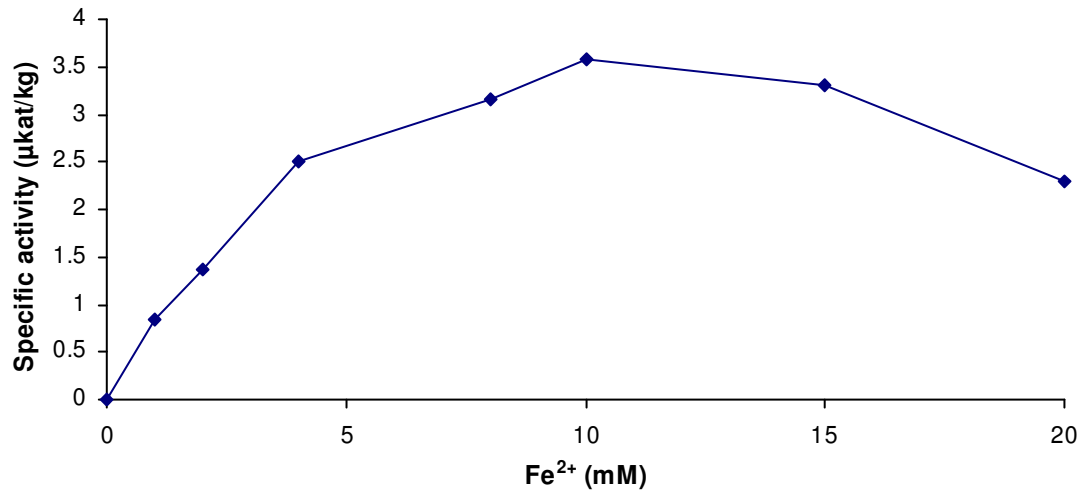


Fig. 4-10: Dependence of dimethylallyltransferase activity on the concentration of Fe^{2+}.

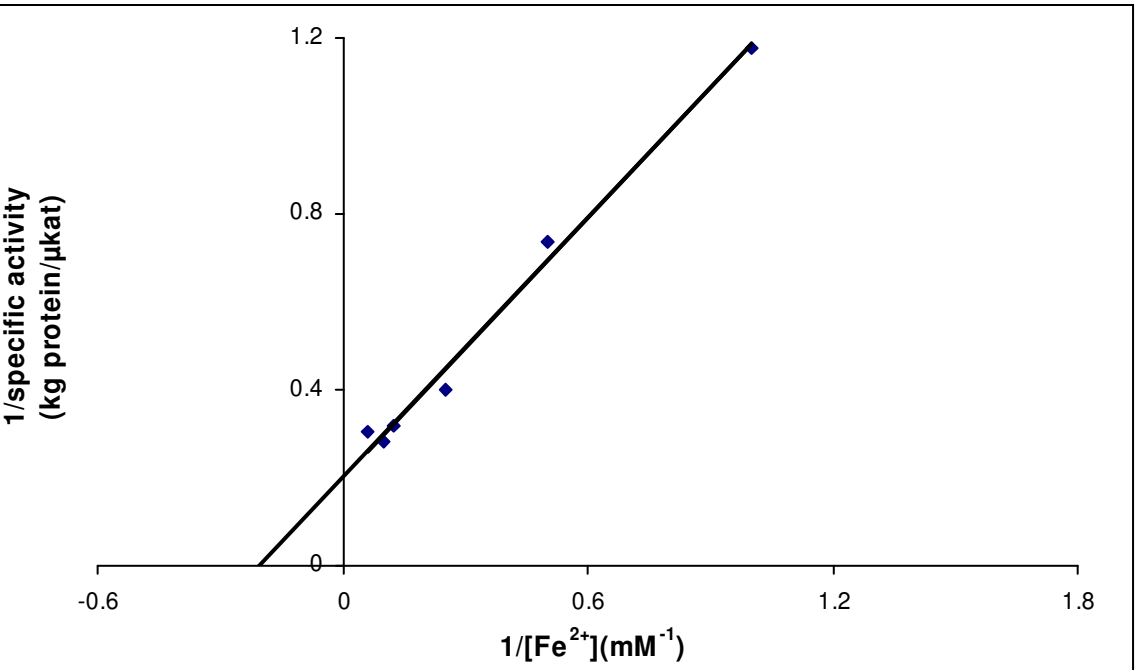


Fig. 4-11: Determination of the K_m value for Fe^{2+} from a Lineweaver-Burk plot.

4.2.5 Substrate specificity

The transferase used DMAPP as the prenyl donor. Product formation also occurred in the presence of IPP, but this was attributed to IPP isomerase activity in the crude extract. No enzyme activity was observed with GPP, FPP, and GGPP as prenyl donors. The preferred prenyl acceptor was phlorisobutyrophenone. High transferase activity was also found with phloracetophenone.
No enzyme activity was observed with phloroglucinol as prenyl acceptor (Table 4-2).

Table 4-2: Substrate specificity of phlorisobutyrophenone dimethylallyltransferase.

Prenyl donor	Rel. enzyme activity (%)	Prenyl acceptor	Rel. enzyme activity (%)
dimethylallyl diphosphate (DMAPP)	100[a]	phlorisobutyrophenone	100[a]
isopentenyl diphosphate (IPP)	30	phloracetophenone	80
geranyl diphosphate (GPP)	< 0.3[b]	phloroglucinol	< 0.3[b]
farnesyl diphosphate (FPP)	< 0.3[b]		
geranylgeranyl diphosphate (GGPP)	< 0.3[b]		

[a] specific activity: 3.6 µkat/kg

[b] Detection limit: 1 ng

4.2.6 Synthesis of the reference dimethylallylphloracetophenone

The expected product from the prenylation of phloracetophenone was dimethylallyl-phloracetophenone (3-(3-methyl-2-butenyl)-2,4,6-trihydroxyacetophenone). Since this compound was not commercially available as a reference, it had to be synthesized chemically (3.1.3). The purified (3.1.3.1) product ($C_{13}H_{16}O_4$) had an absorption maximum at 291 nm (Fig. 4-12) and a molar mass of 236 g/mol (Fig. 4-13).

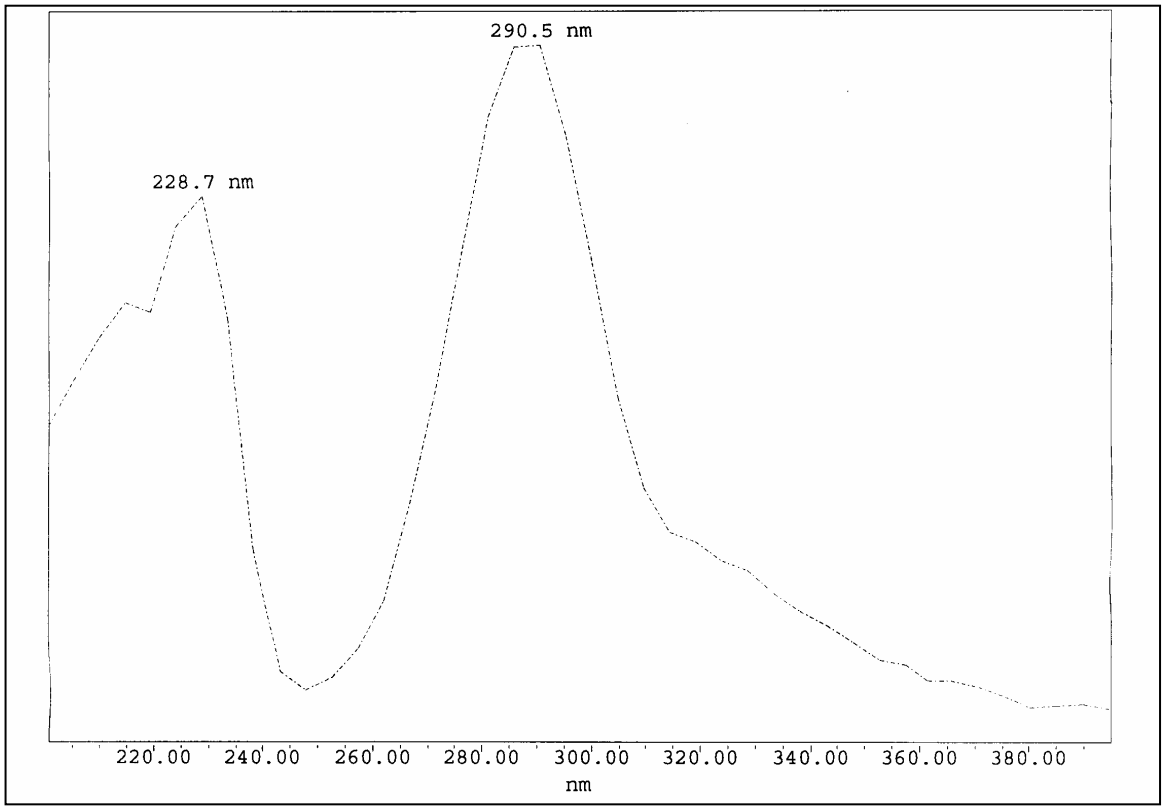


Fig. 4-12: UV spectrum of the synthetic reference dimethylallylphloracetophenone

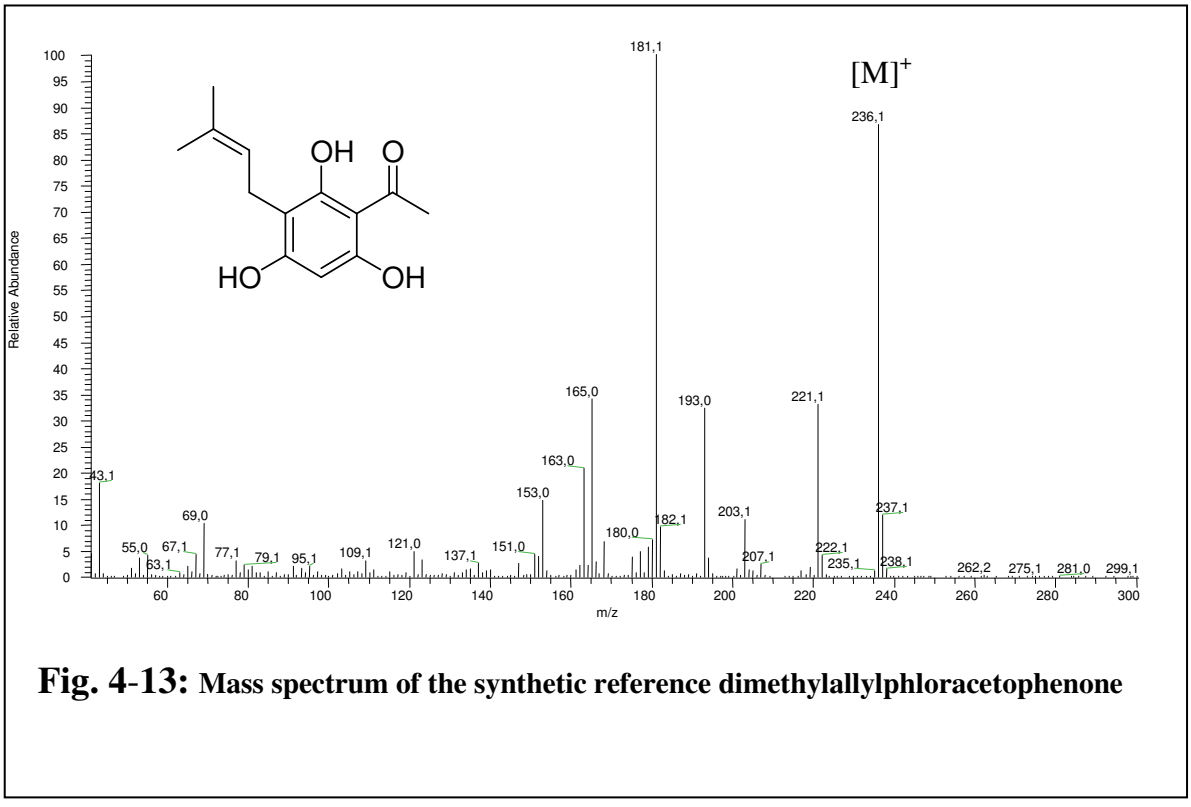


Fig. 4-13: Mass spectrum of the synthetic reference dimethylallylphloracetophenone

The ^{1}H NMR spectrum of dimethylallylphloracetophenone is shown in Fig. 4-14.

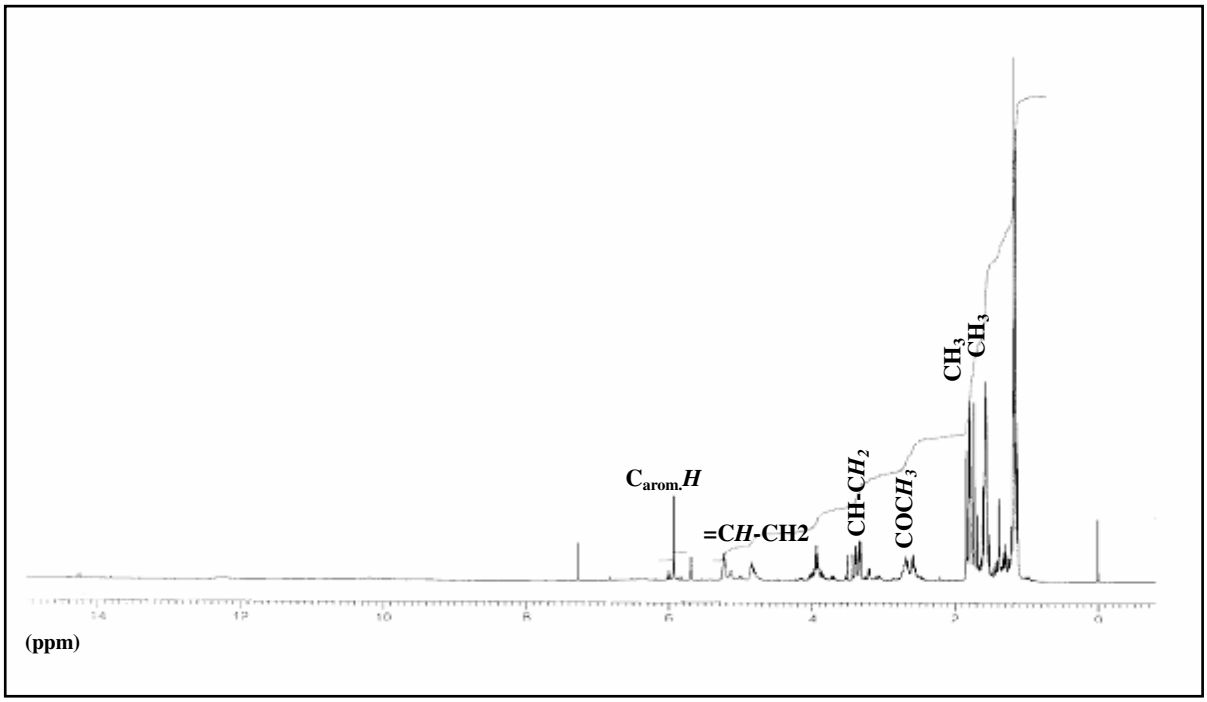


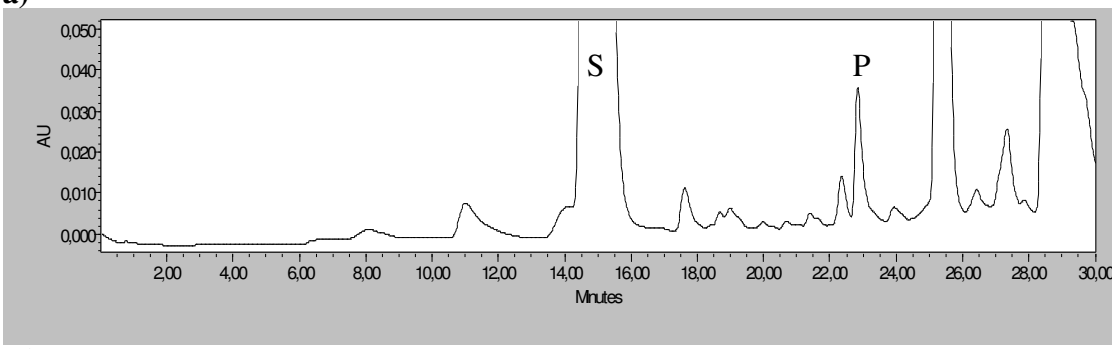
Fig. 4-14: ^{1}H NMR spectrum of the synthetic reference dimethylallylphloracetophenone

The spectroscopic data of 3-(3-methyl-2-butenyl)-2,4,6-trihydroxyacetophenone corresponded to published data (Jakupovic et al., 1986). They are summarized as follows: $R_{f.}$ (silica gel, diethyl ether : pentane 60 : 40) 0.44; RI (ZB5) 2177; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.78 (3 H, s, CH_3), 1.83 (3 H, s, CH_3), 2.66 (3 H, s, $COCH_3$), 3.36 (2 H, d, J=7.2 Hz, CH-CH_2), 5.25 (1H, m, =C*H*-CH2), 5.83 (1 H,s, $C_{arom.}H$); EIMS, 70eV, m/z (rel. int.): 181 (100, $[M-C_4H_7]^+$), 236 (88, $[M]^+$), 165 (34), 221 (32, $[M-CH_3]^+$), 193 (32, $[M-CH_3CO]^+$), 163 (20), 43 (18), 153 (14), 203 (11), 182 (9), 69 (9),168 (7).

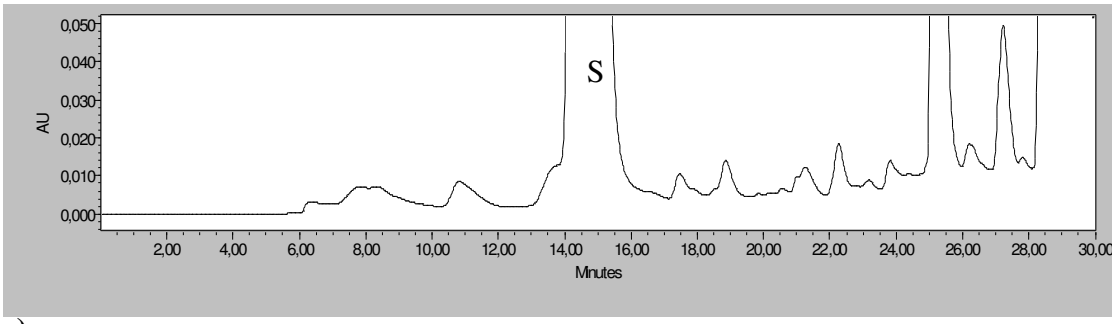
4.2.7 Analysis of the enzymatic product dimethylallylphloracetophenone

The product resulting from the monoprenylation of phloracetophenone was identified as dimethylallylphloracetophenone by HPLC (Fig. 4-15) in comparison with a sample of the synthesized reference compound. The enzymatic product was extracted from large-scale incubations, purified by TLC and analyzed by GC-MS (Fig. 4-16). The synthetic reference compound and the enzymatic product exhibited identical mass spectra and retention times.

a)



b)



c)

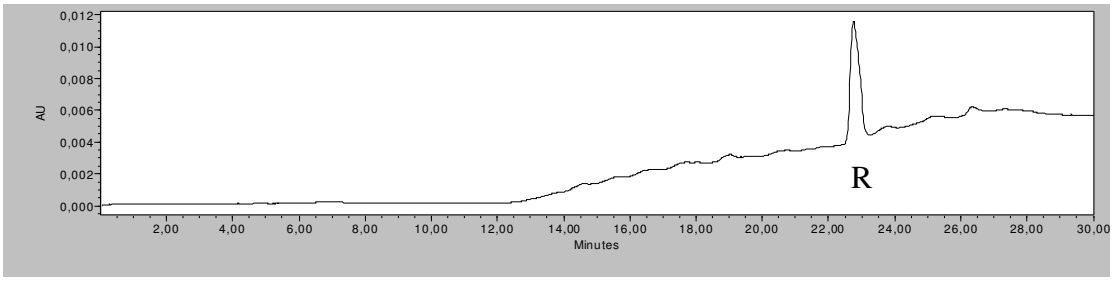


Fig. 4-15: HPLC analysis of prenyltransferase assays with phloracetophenone. Detection wavelength 291 nm

a) Standard assay

b) Incubation with denatured protein

c) Reference compound dimethyallylphloracetophenone

P = Enzymatic product (dimethylallylphloracetophenone)
S = Substrate (phloracetophenone)
R = Reference compound

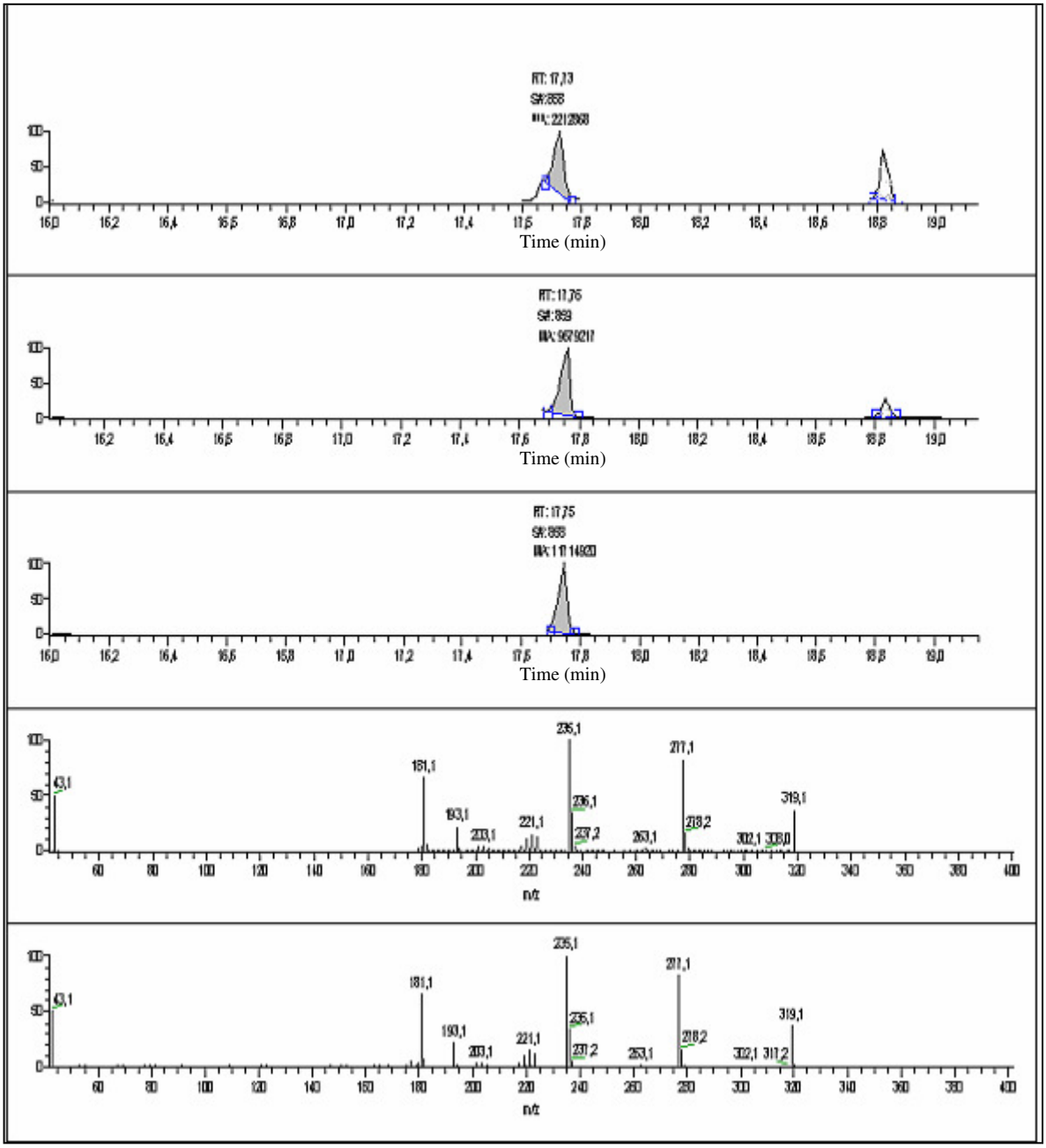


Fig. 4-16: GC-MS analysis of the enzymatic product dimethylallylphloracetophenone and the synthetic reference compound (after acetylation). GC of enzymatic product (a) and reference compound (c), Co- chromatography (b). Mass spectra of the enzymatic product (d) and the reference compound (e).

4.2.8 Determination of kinetic data

Prenylation of phlorisobutyrophenone followed Michaelis-Menten kinetics and the K_m values were calculated from Lineweaver-Burk plots.

For phlorisobutyrophenone, the concentration varied between 0.1 and 8 mM while the assays contained 2 mM DMAPP (Fig.4-17). The K_m value was 0.52 mM (Fig. 4-18). Two independent experiments were performed and mean values calculated.

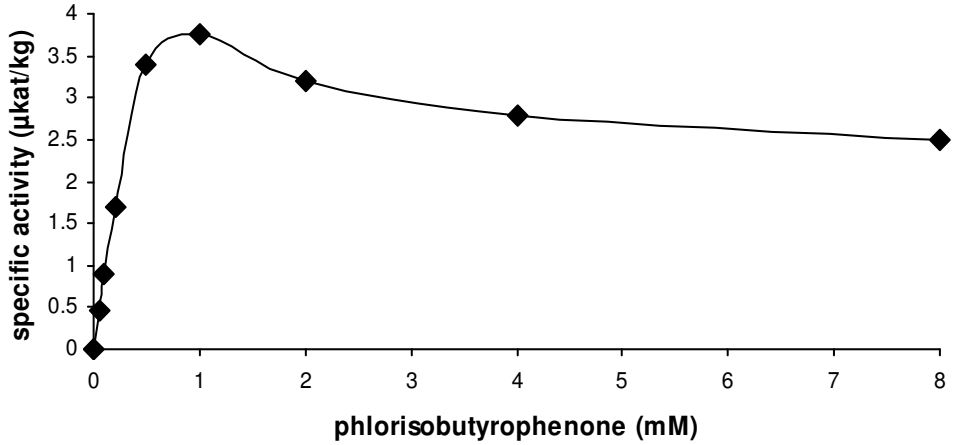


Fig. 4-17: Dependence of dimethylallyltransferase activity on the concentration of phlorisobutyrophenone.

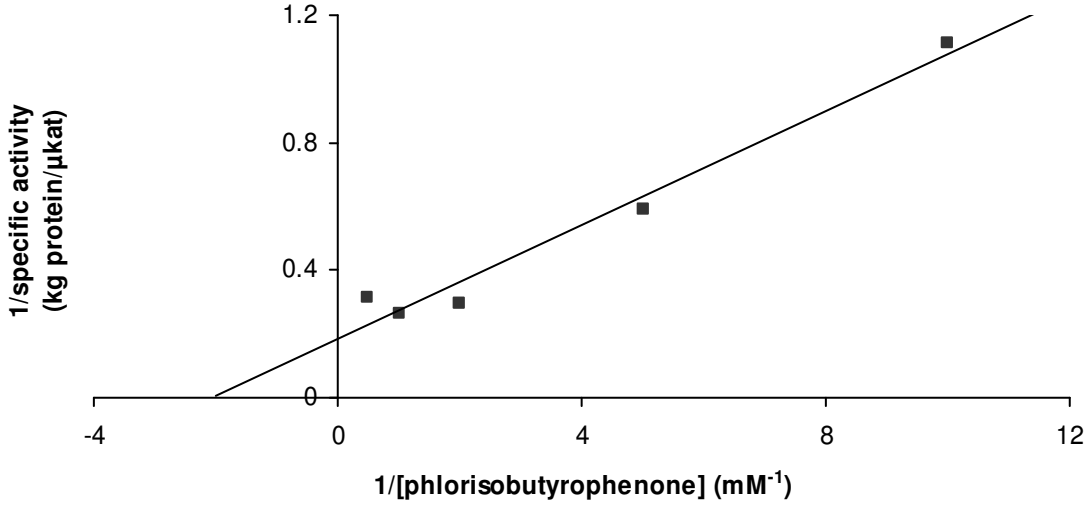


Fig. 4-18: Determination of the K_m value for phlorisobutyrophenone from a Lineweaver-Burk plot.

For DMAPP, the concentration was changed between 0.2 and 16 mM while that of phlorisobutyrophenone was kept constant at 2 mM (Fig. 4-19). The increase in prenyltransferase activity obeyed Michaelis-Menten kinetics. High DMAPP concentration inhibited the enzyme. These data points were not included in the Lineweaver-Burk plot. The K_m value was 0.46 (Fig. 4-20). Two independent experiments were performed and mean values calculated.

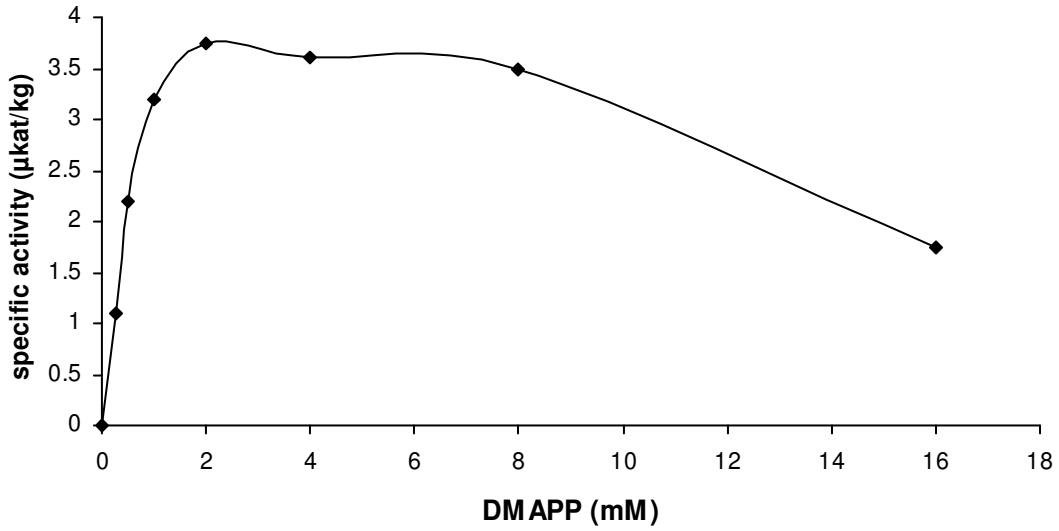


Fig. 4-19: Dependence of dimethylallyltransferase activity on the concentration of DMAPP.

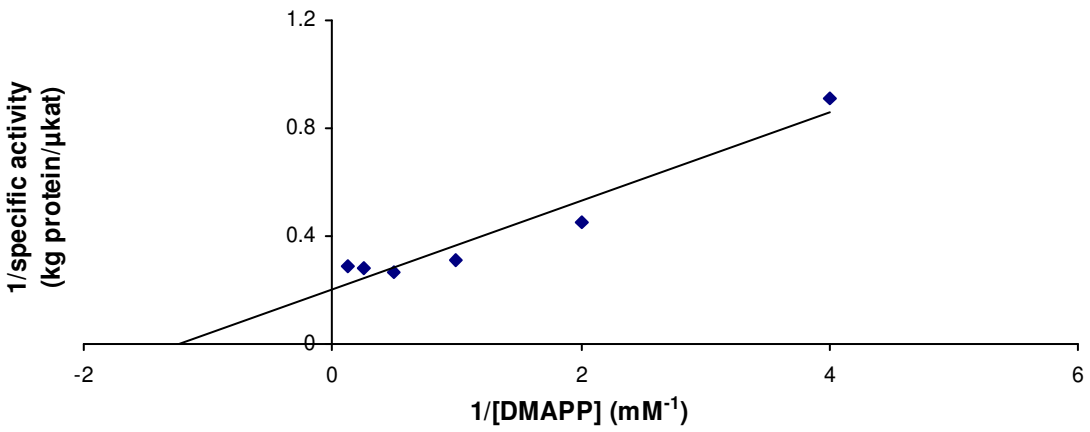


Fig. 4-20: Determination of the K_m value for DMAPP from a Lineweaver-Burk plot.

4.2.9 Prenyltransferase activity in cell fractions after ultracentrifugation

After ultracentrifugation, 80 % of the enzyme activity was found in the supernatant (Table 4-3). The specific activity in the supernatant was more than 20 times that in the pellet. These data indicate that the enzyme involved in hyperforin biosynthesis is a soluble dimethylallyltransferase.

Table 4-3: Prenyltransferase activity in cell fractions after ultracentrifugation.

Cell fraction	Protein (mg)	Total activity pkat	%	Specific activity (µkat /kg)
Crude cell-free extract	5.5	19.64	100	3.57
Supernatant (100 000 g)	4.9	15.74	80	3.213
Pellet (100 000 g)	0.5	0.0714	0.36	0.1428

4.2.10 Changes in prenyltransferase activity and adhyperforin formation during cell culture growth

The accumulation of hyperforins in *H. calycinum* cell cultures occurred between day 3 and 6 (Klingauf et al., 2005). It was preceded by an increase in dimethylallyltransferase activity. The maximum specific enzyme activity was 3.6 µkat/kg protein and observed at day 3 (Fig. 4-14). Thereafter, the enzyme activity decreased and declined completely during the stationary phase.

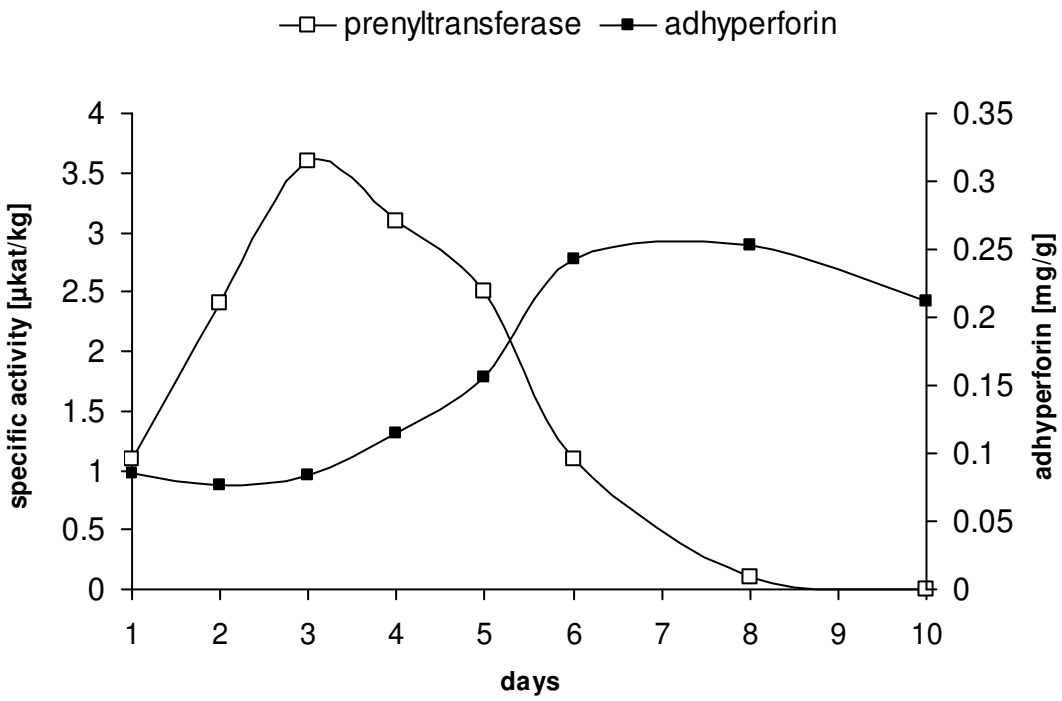


Fig. 4-21: Adhyperforin formation and changes in prenyltransferase activity during cell culture growth. Values are means of two independent experiments.

4.3. Purification of the prenyltransferase

4.3.1 Fractionation by ammonium sulphate precipitation

The majority of prenyltransferase activity was precipitated between 30 and 65% saturation (Fig. 4-22). This fraction was used for subsequent column chromatographies.

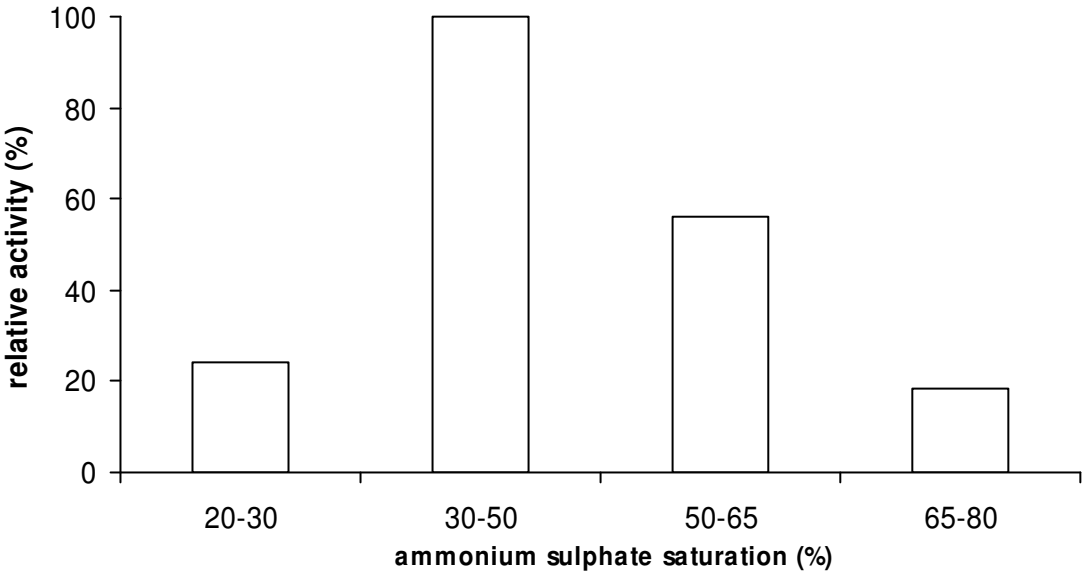


Fig. 4-22: Distribution of prenyltransferase activity in ammonium sulphate-precipitated protein fractions.

4.3.2 Column chromatography

The protein fraction precipitating between 30 and 65% ammonium sulphate saturation was subjected to various column chromatographies in order to test the purification efficiency of these procedures.

4.3.2.1 DEAE-anion exchange chromatography

4.3.2.1.1 DEAE-column with 1 ml gel volume

Ion exchange chromatography was preceded by ammonium sulphate precipitation as the first purification step. The protein fraction precipitating between 30 and 65% ammonium sulphate saturation was loaded onto a DEAE-Sepharose column with 1 ml gel volume, after the buffer

had been exchanged for DEAE starting buffer. Bound proteins were eluted with a linear salt gradient from 0-0.5 M KCl. The protein elution profile is shown in Fig. 4-23. Fractions 1-7 are related to sample application and removal of unbound proteins. Fractions 8-24 contain bound proteins.

Measuring prenyltransferase activity in the individual fractions (Fig. 4-24) showed that the enzyme eluted between 0.3 and 0.4 M KCl (fractions 19-22), with the major fraction in the activity profile (fraction 21) being eluted at 0.35 M KCl.

Fractions 19-22 that contained high prenyltransferase activity were pooled and used for the subsequent purification step.

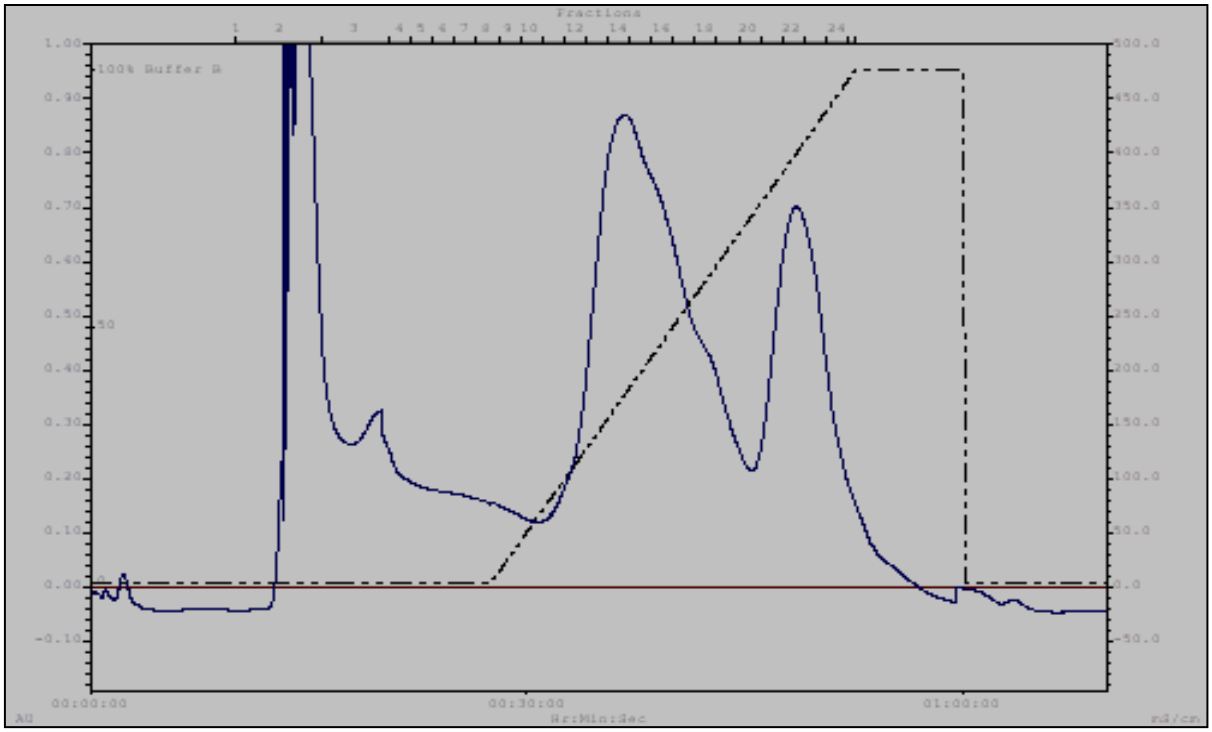


Fig. 4-23: Anion exchange chromatography of the protein fraction precipitating between 30 and 65% ammonium sulphate saturation on a 1 ml-DEAE-Sepharose column (Protein was measured at 280 nm)

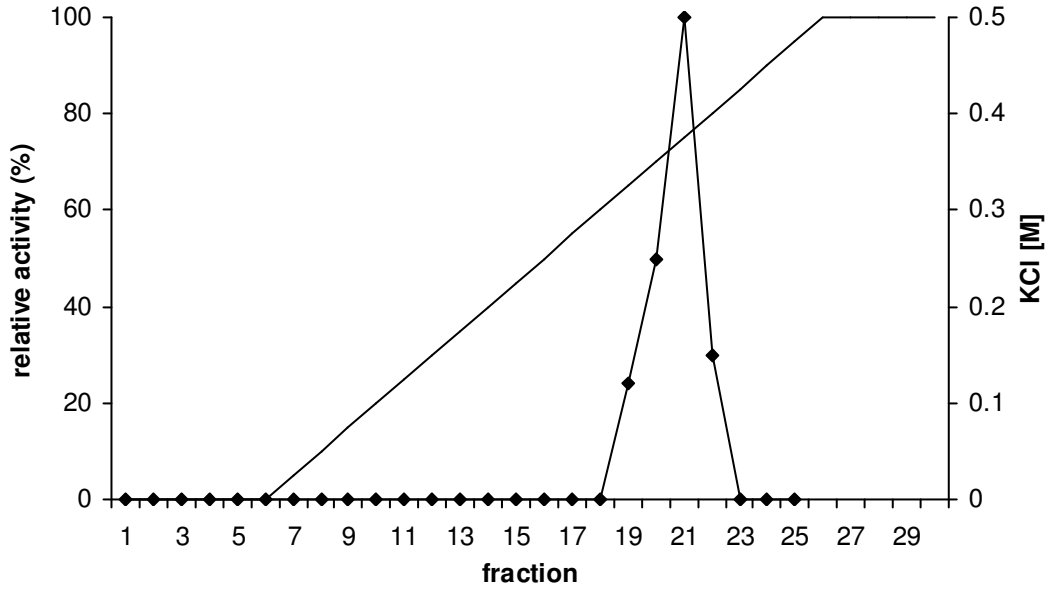


Fig. 4-24: Detection of prenyltransferase activity in protein fractions eluted from the DEAE-Sepharose column (Fig. 4-23)

4.3.2.1.2 DEAE-column with 5 ml gel volume

The use of a larger DEAE-Sepharose column (5 ml) in the first step of the purification scheme provided a convenient method to load larger quantities of the 30 to 65% ammonium sulphate precipitated protein fraction and to improve the recovery. The procedure used corresponded to that described above. Prenyltransferase activity was detected in the fractions eluting between 0.3 and 0.4 M KCl.

4.3.2.2 Hydroxylapatite-adsorption chromatography

The prenyltransferase-containing protein fraction precipitating between 30 and 65% ammonium sulphate saturation was subjected to buffer exchange and subsequently loaded onto a hydroxylapatite column (Bio-Rad CHT5-1) equilibrated with starting buffer. Elution was carried out with a linear gradient from 10-500 mM phosphate buffer pH 7.5. Bound proteins were eluted between 60 and 250 mM phosphate (Fig. 4-25). After measuring the prenyltransferase activity in each fraction, the activity profile (Fig. 4-26) showed that most of the enzyme activity was eluted between 120 and 200 mM phosphate. Fractions showing the highest prenyltransferase activity were pooled and used for the subsequent purification step.

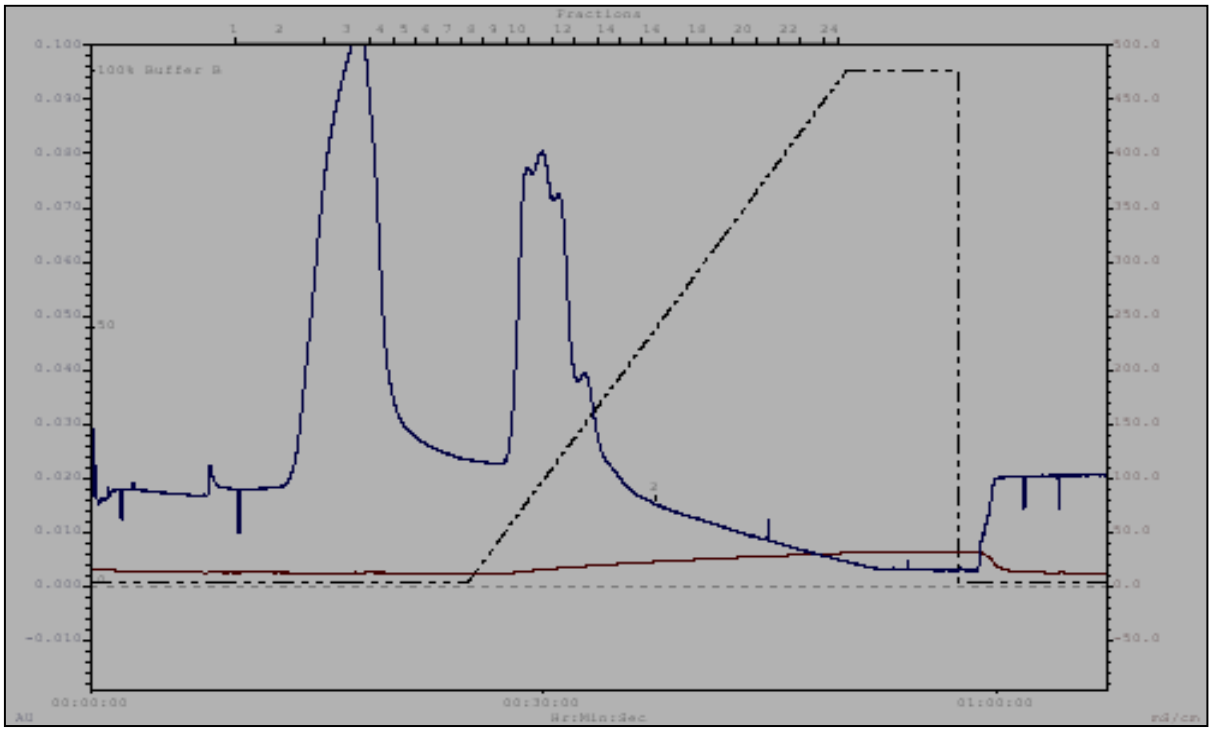


Fig. 4-25: Hydroxylapatite-adsorption chromatography of the protein fraction precipitating between 30 and 65% ammonium sulphate saturation on a CHT5-1 column (Protein was measured at 280 nm)

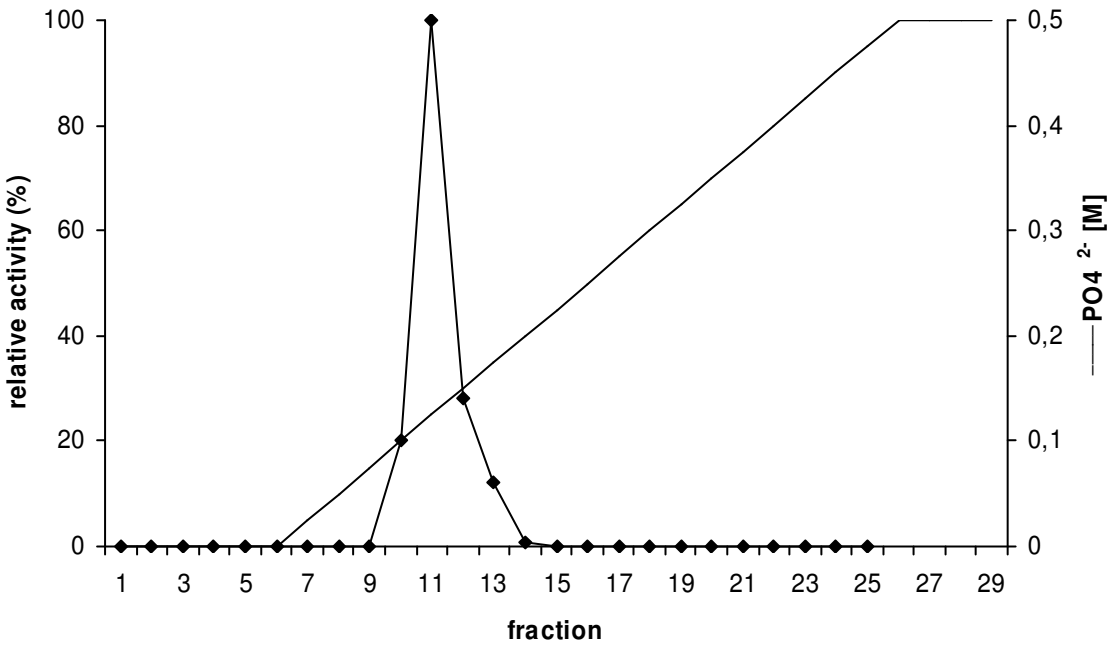


Fig. 4-26: Detection of prenyltransferase activity in protein fractions obtained by hydroxylapatite-adsorption chromatography (Fig. 4-25)

4.3.2.3 Hydrophobic interaction chromatography

Protein from ammonium sulphate fractionation (30 to 65% saturation) was passed through a hydrophobic interaction chromatography column (Phenyl-Sepharose). The sample was loaded at a high salt concentration and elution was performed using a descending gradient (Fig. 4-27). Proteins with low hydrophobicity did not absorb to the hydrophobic resin and therefore were eluted during the washing step (fractions 1-12). Bound proteins were eluted in roughly two peaks by decreasing the ammonium sulphate concentration in a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in phosphate buffer pH 7.5.

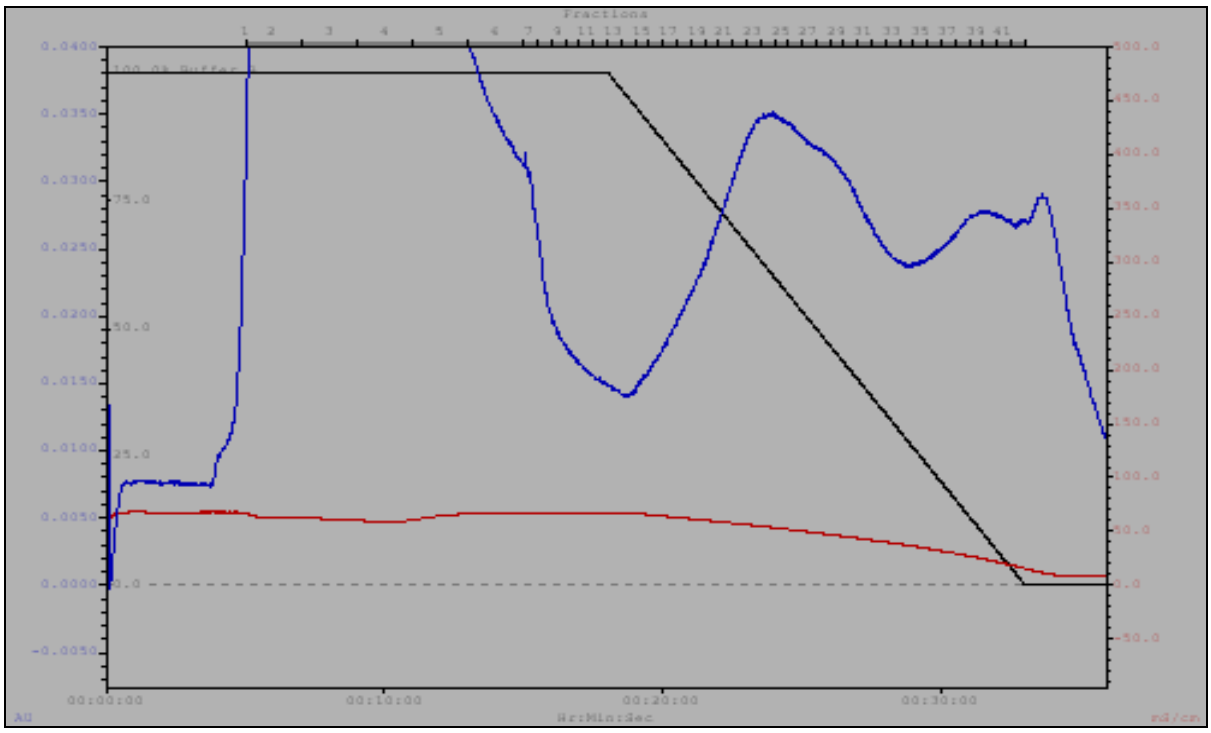


Fig. 4-27: Hydrophobic interaction chromatography of the protein fraction precipitating between 30 and 65% ammonium sulphate saturation on a Phenyl- Sepharose column (Protein was measured at 280 nm)

Prenyltransferase activity was eluted in the first peak of the descending salt gradient (0.8-0.7 M $(NH_4)_2SO_4$). Other components were more tightly bound to the hydrophobic resin and only eluted with buffer containing low ammonium sulphate concentrations.

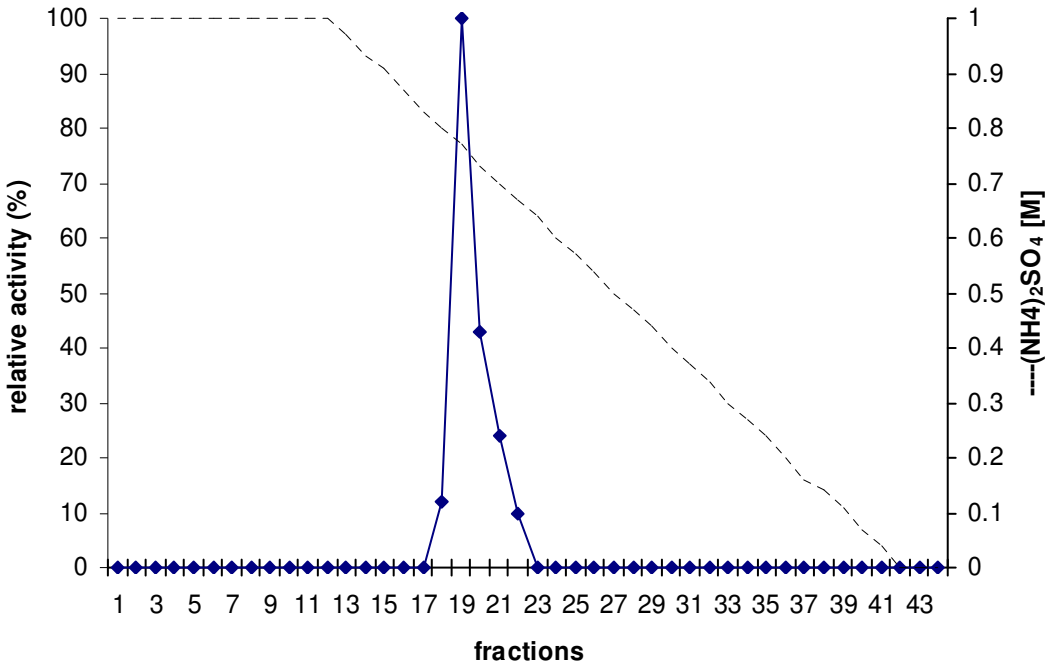


Fig. 4-28: Detection of prenyltransferase activity in protein fractions separated on a Phenyl-Sepharose column (Fig. 4-27)

4.3.2.4 Mono-Q anion exchange chromatography

Protein from ammonium sulphate fractionation (30 to 65% saturation) was passed through a Mono-Q anion exchange column after the buffer had been replaced by Mono-Q starting buffer. The profile of protein elution from the Mono-Q column is shown in Fig. 4-29. Bound proteins were eluted in two peaks using a linear KCl gradient from 0 to 0.5 M KCl. The gradient began at fraction 8 and reached 0.5 M KCl in fraction 25.

The activity profile obtained by assaying enzyme activity in the individual fractions (Fig. 4-30) showed that the prenyltransferase eluted in the second, smaller protein peak at KCl concentrations between 0.33 and 0.38 M (fractions 19-21). These fractions were pooled and used for further enzyme purification.

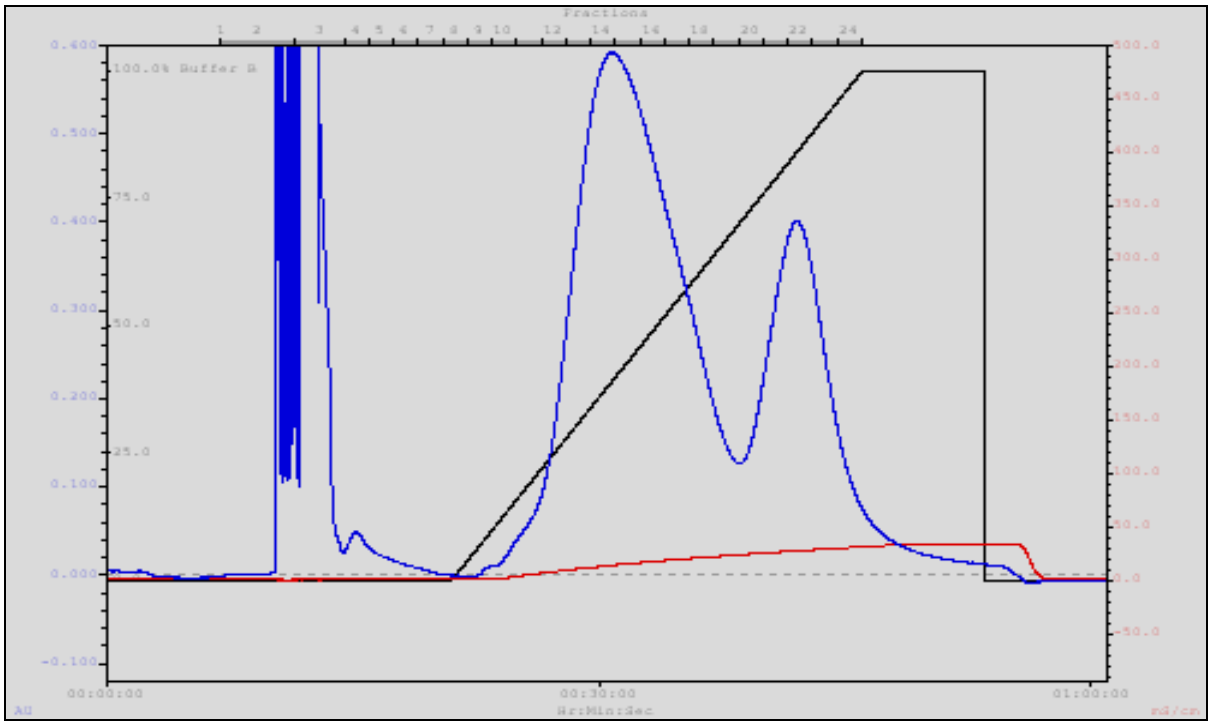


Fig. 4-29: **Anion exchange chromatography of the protein fraction precipitating between 30 and 65% ammonium sulphate saturation on a Mono-Q column (Protein was measured at 280 nm)**

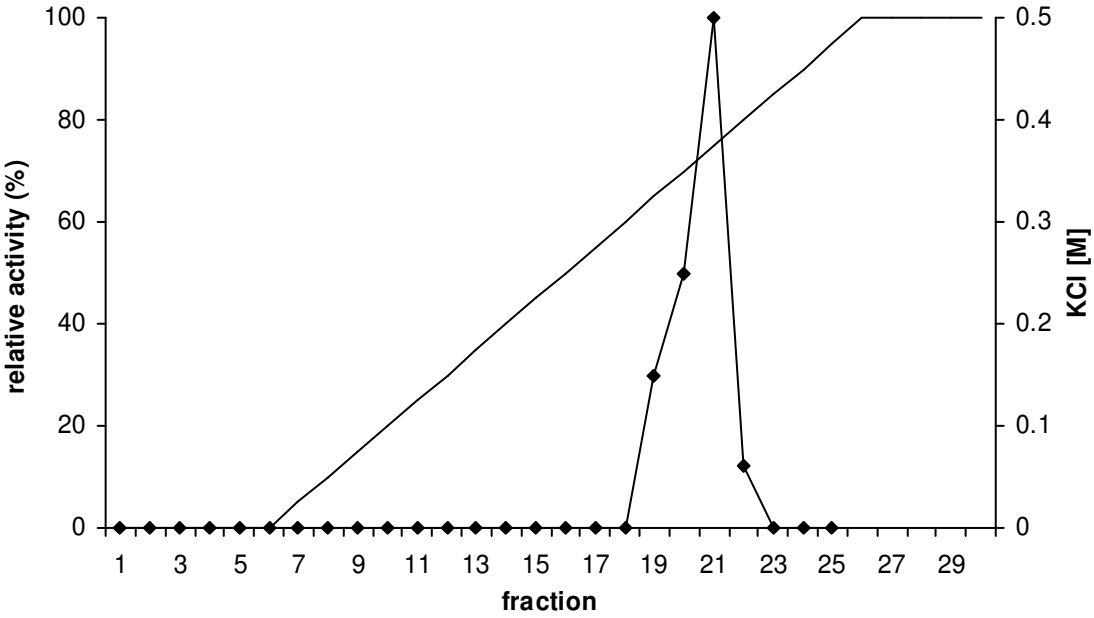


Fig. 4-30: **Detection of prenyltransferase activity in protein fractions eluted from a Mono-Q column**

4.3.2.5 Gel filtration

Chromatography on a gel filtration column was used for prenyltransferase purification as well as determination of the native molecular mass (4.3.5.1). A Sephacryl 200 HR column was calibrated using blue dextran and the standard proteins cytochrome c, carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase and β-amylase. Elution of blue dextran was used to measure the void volume (V_o), as shown in Fig. 4-31. The elution volumes (V_e) of the standard proteins were determined, as depicted in Fig. 4-32. To obtain a calibration curve, the molecular masses of the markers were plotted versus the V_e / V_o ratio (Fig. 4-33).

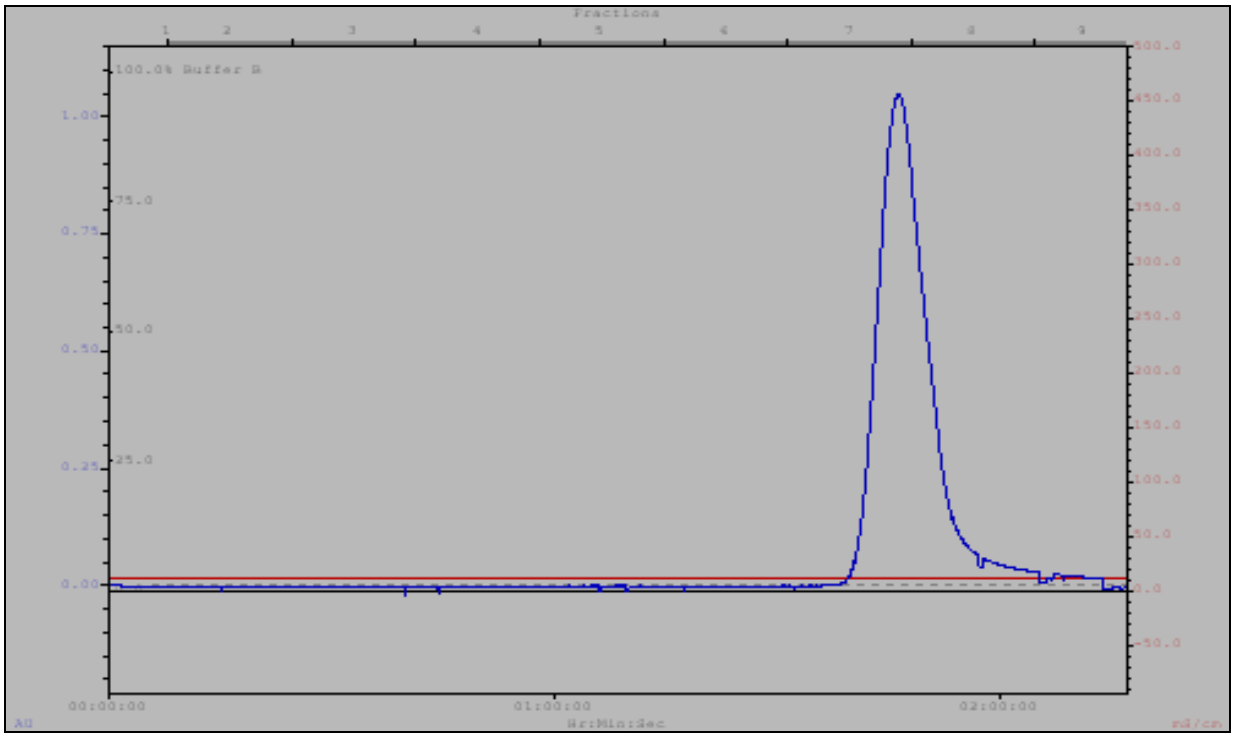


Fig. 4-31: Elution profile of blue dextran on a Sephacryl S-200 HR column (Protein was measured at 280 nm)

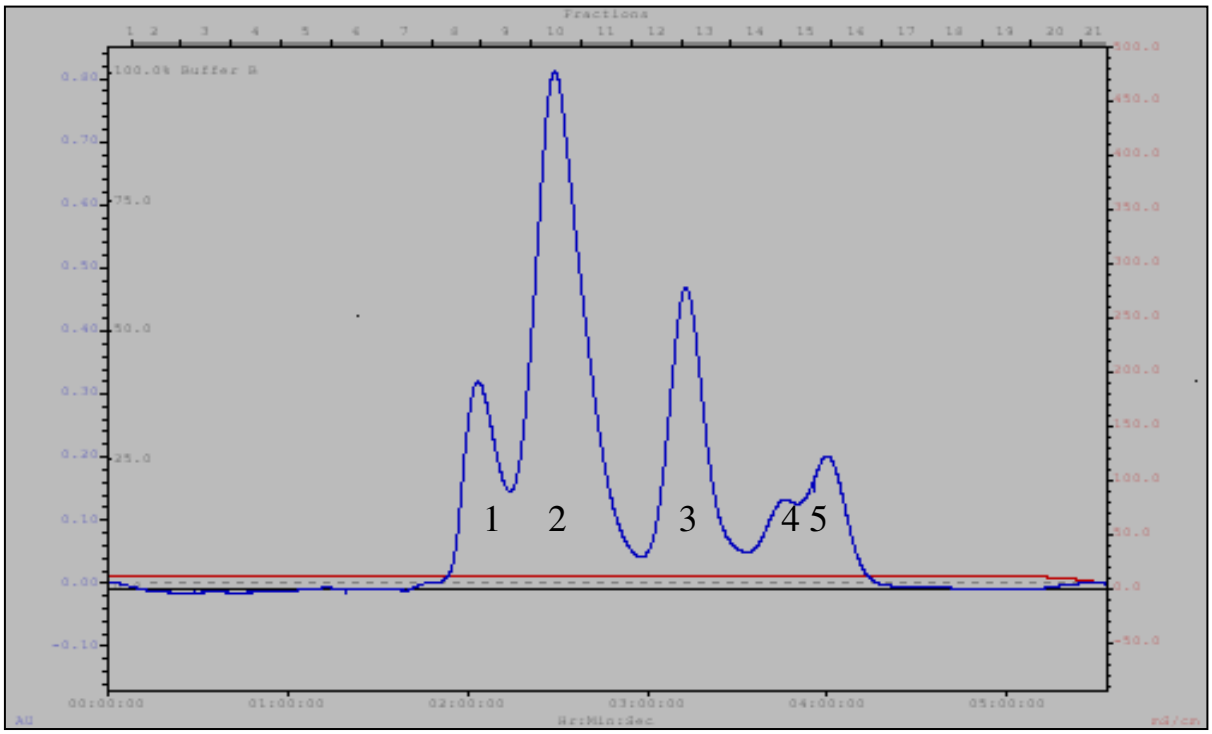


Fig. 4-32: Elution profile of standard proteins on a Sephacryl S-200 HR column (Protein was measured at 280 nm)
1: β-amylase (200 kDa), 2: alcohol dehydrogenase (150 kDa), 3: albumin (66 kDa), 4: carbonic anhydrase (29 kDa), 5: cytochrome c (12.4 kDa)

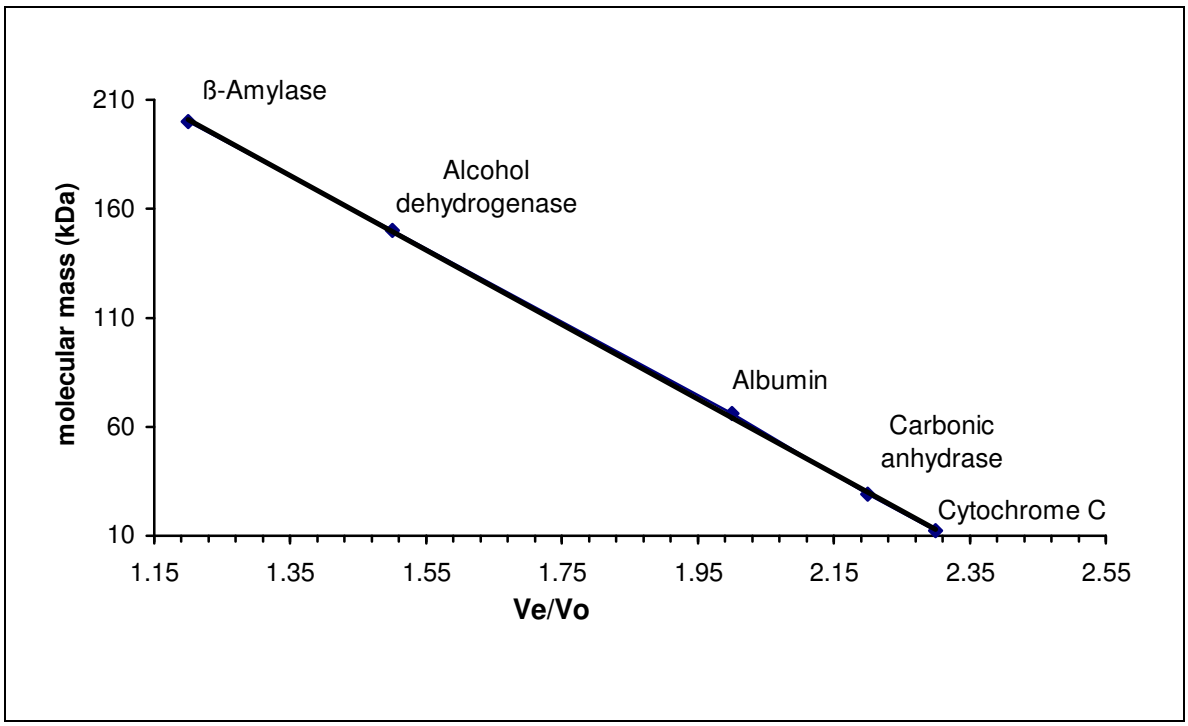


Fig. 4-33: Calibration of a Sephacryl S-200 HR column

4.3.3 Purification of the prenyltransferase

The chromatographic steps described above were combined as shown in Fig. 4-34. After each step, the active fractions were localized by measuring prenyltransferase activity, then pooled and passed through PD10 columns equilibrated with the starting buffer for the next column.

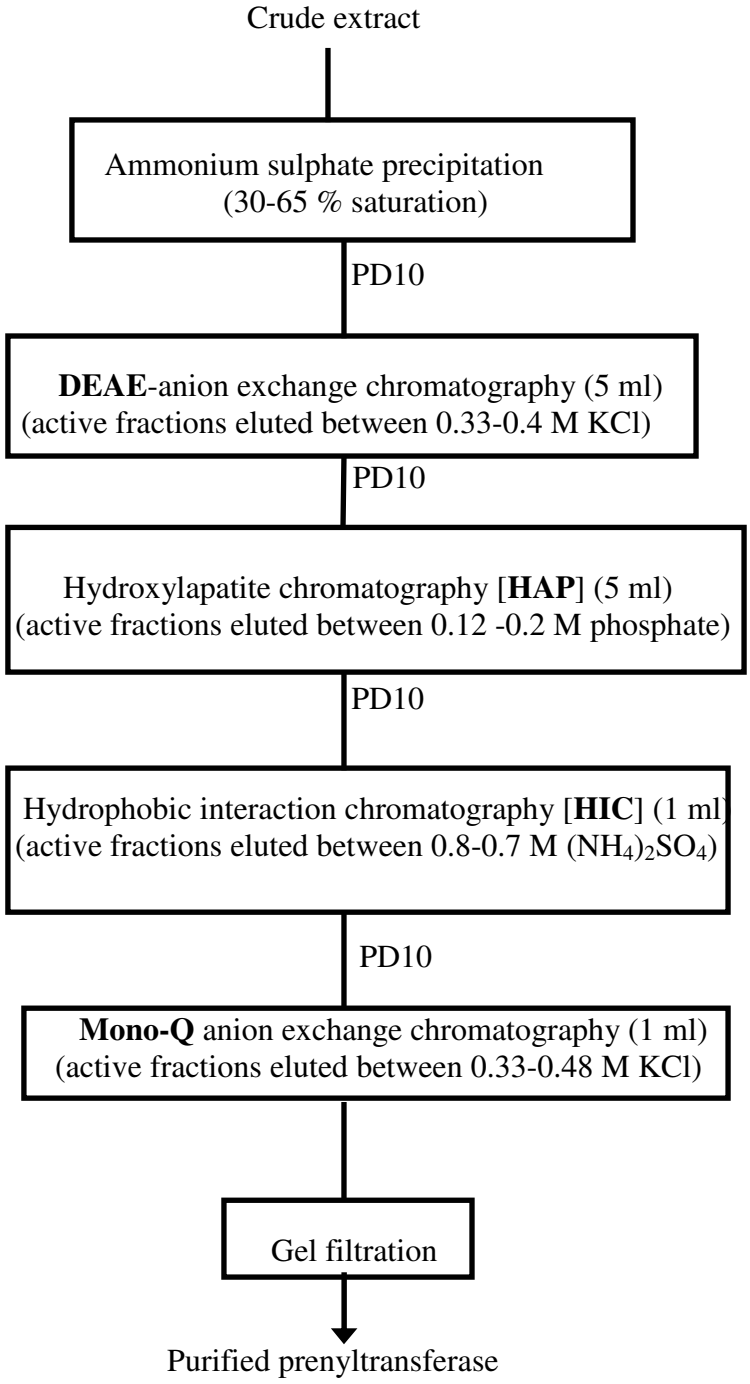


Fig. 4-34: Diagram depicting the purification scheme applied to the prenyltransferase from *H. calycinum* cell cultures

For the final purification step, i.e. gel filtration, the active fractions after chromatography on the Mono-Q column were pooled and loaded onto a Sephacryl S-200 column. Three major protein peaks were eluted (Fig. 4-35). Prenyltransferase activity was located in the third peak (fraction 12). The fractions 1-11 lacked prenyltransferase activity. The protein-containing fractions were analyzed by SDS-PAGE in a 12% gel and protein bands were visualized by silver staining. Fraction 12 which contained prenyltransferase activity exhibited the highest grade of purity (Fig. 4-37).

These results suggested that the prenyltransferase could be purified to apparent homogeneity using fractionating ammonium sulphate precipitation, ion-exchange chromatography on DEAE-Sepharose, hydroxylapatite adsorption chromatography, Phenyl-Sepharose hydrophobic interaction chromatography and gel-filtration.

Table 4-4 summarizes the results obtained in the six step-procedures for prenyltransferase purification. After each chromatographic step, the protein content and the prenyltransferase activity were measured and the specific prenyltransferase activity was calculated. The specific enzyme activity in the initial crude extract (step 1) was 3.1 µkat/kg. After ammonium sulphate precipitation (step 2) which allowed concentration of the cell-free extract, it increased 2.5-fold with about 91% recovery of enzyme activity. After gel-filtration (step7), the recovery of preanyltransferase activity was 0.29%, accompanied by a 65-fold increase in the specific activity (200 µkat/kg). Hydrophobic interaction chromatography on the Phenyl-Sepharose column (step 5) was a highly efficient purification step because the majority of contaminants passed through the column without being bound. Although a considerable portion of the enzyme activity was lost when loading the column (from 0.54 to 0.18 nkat), the specific activity enhanced from 22 to 127.3 µkat/kg protein and thus the purification factor dramatically increased from 7.1 to 41.1.

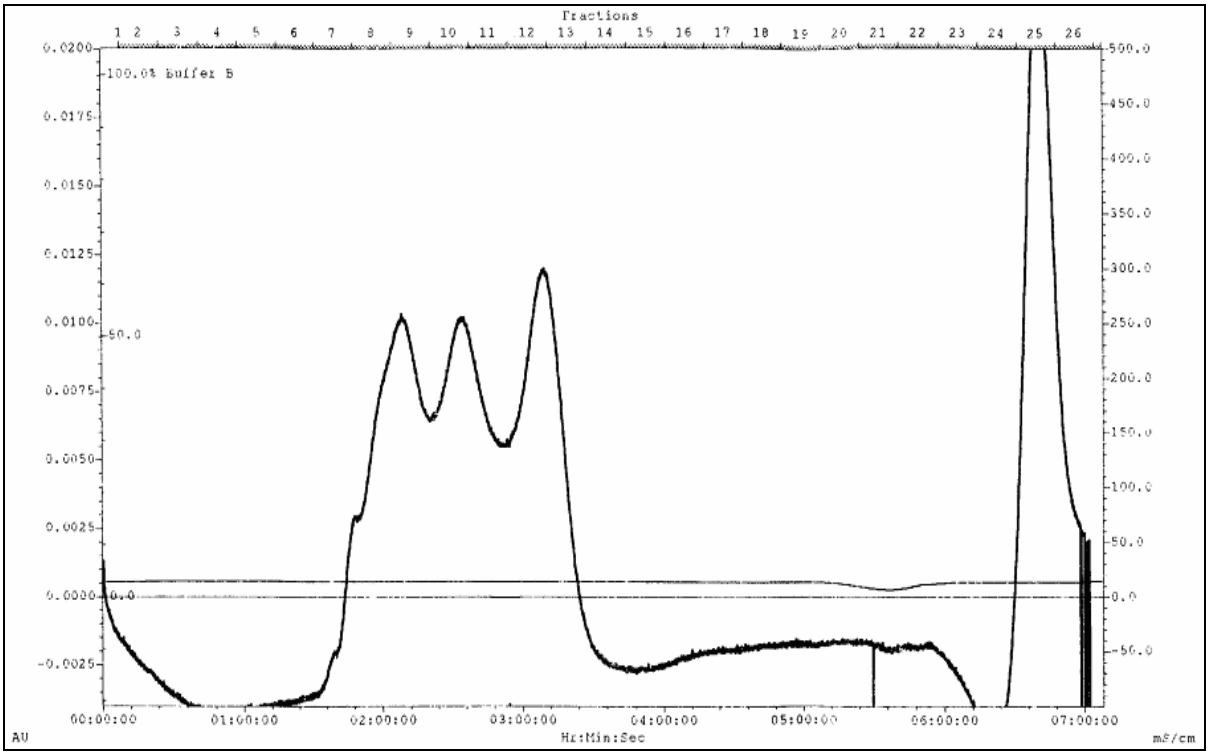


Fig. 4-35: Gel filtration on Sephacryl S-200 of the prenyltransferase-containing protein fraction obtained by Mono-Q anion exchange chromatography (Protein was measured at 280 nm)

Tab. 4-4: Purification of the prenyltransferase from *H. calycinum* cell cultures

Purification Step	Total protein (mg)	Total activity (nkat)	Specific activity (µkat/kg)	Purification (-fold)	Yield (%)
Crude extract	1736[a]	5.4	3.1	1	100
$(NH_4)_2SO_4$ (30-65 %)	633.4	4.9	7.7	2.5	91
DEAE	124.9	2.2	17.6	5.7	41
HAP	24.6	0.54	22	7.1	10
HIC	1.4	0.18	127.3	41.1	3.3
Mono-Q	0.39	0.07	182.8	59	1.32
Gel filtration	0.1	0.02	200	65	0.29

[a] from 1379 g fresh weight

4.3.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The degree of purification of the prenyltransferase after each chromatographic step was examined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the subunit molecular mass of the prenyltransferase was determined (4.3.5.2).

4.3.4.1 Preparation of samples

Aliquots of the active, pooled fractions after each step in the purification sequence were analyzed. After gel filtration, aliquots of the protein-containing fractions (10 µg each) were concentrated and subjected to SDS-PAGE.

4.3.4.2 Electrophoresis and detection of protein bands

Proteins were visualized using silver staining. Fig. 4-36 shows the complexity of the protein fractions at different stages of prenyltransferase purification. Initially, the samples contained a heterogeneous mixture of proteins (lanes 1-6). In the course of purification, the number of protein bands gradually decreased. The efficiency of gel filtration is evident when lanes 6 and 7 are compared. After gel filtration, two closely spaced protein bands were observed with estimated molecular masses of ~ 31 kDa (Fig. 4-36 and 4-37B). These two polypeptides were absent from the other fractions analyzed after gel filtration (Fig. 4-37 A).

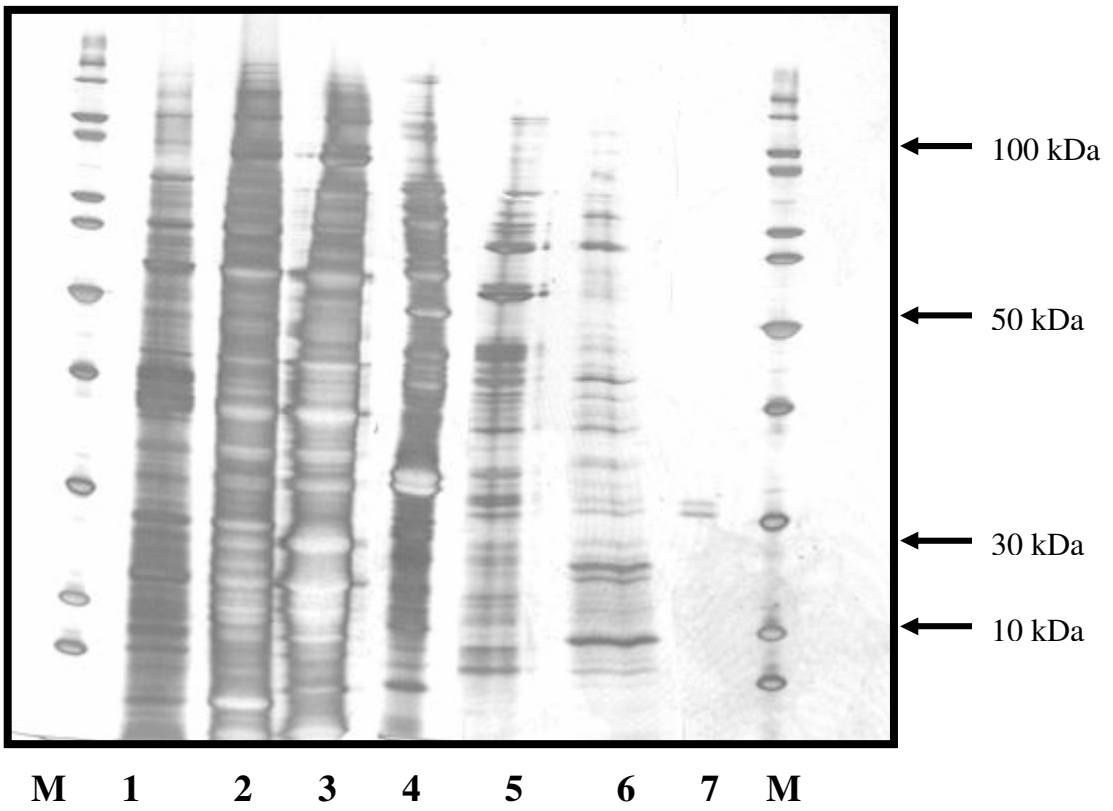


Fig. 4-36: SDS-PAGE (12% gel) of fractions containing prenyltransferase activity after the following purification steps. (1) Crude extract; (2) $(NH_4)_2SO_4$ precipitation; (3) DEAE; (4) HAP; (5) HIC; (6) Mono-Q; (7) Gel filtration. (M) Molecular mass markers. Visualization by silver staining.

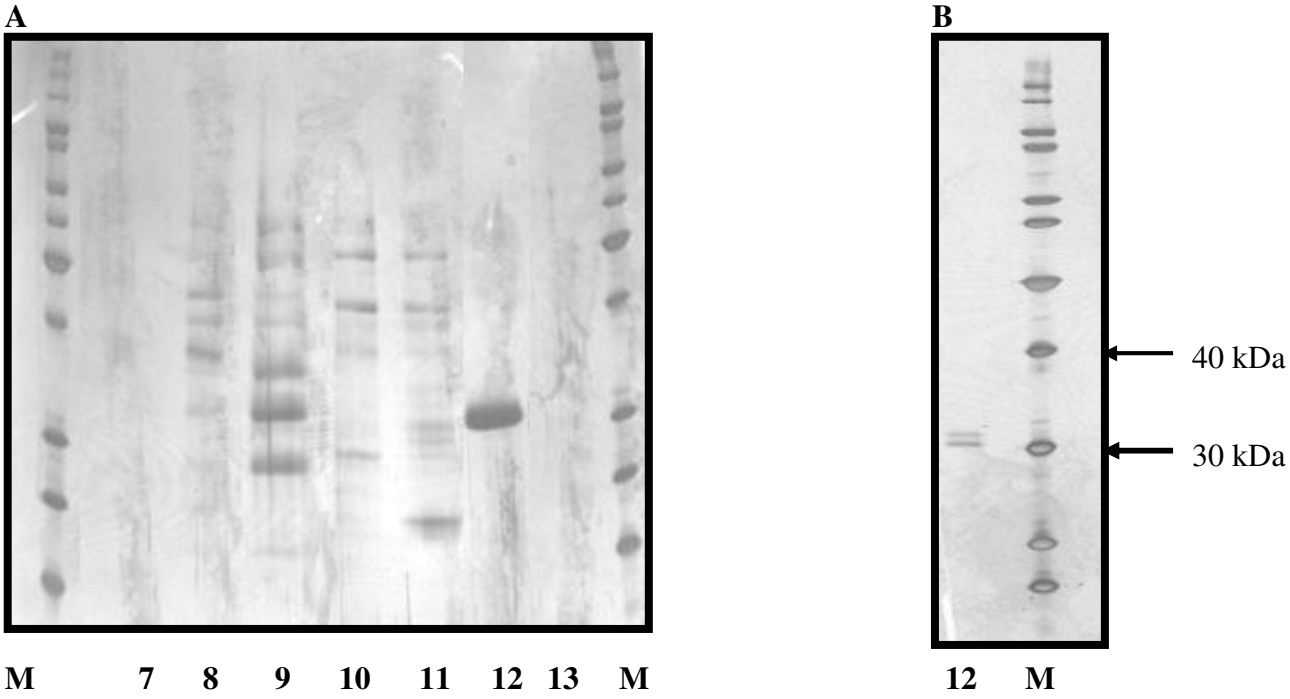


Fig. 4-37: SDS-PAGE of the protein-containing fractions after gel filtration (A). Separation of a small protein quantity from fraction 12 (B).

4.3.5 Determination of the molecular masses

4.3.5.1 Determination of the subunit molecular mass by SDS-PAGE

The migration of the standard proteins (Fig. 4-37B) was used to derive a calibration curve (Fig. 4-38). The relative electrophoretic mobility of the purified protein bands indicated subunit molecular masses of ~ 31 and 31.5 kDa.

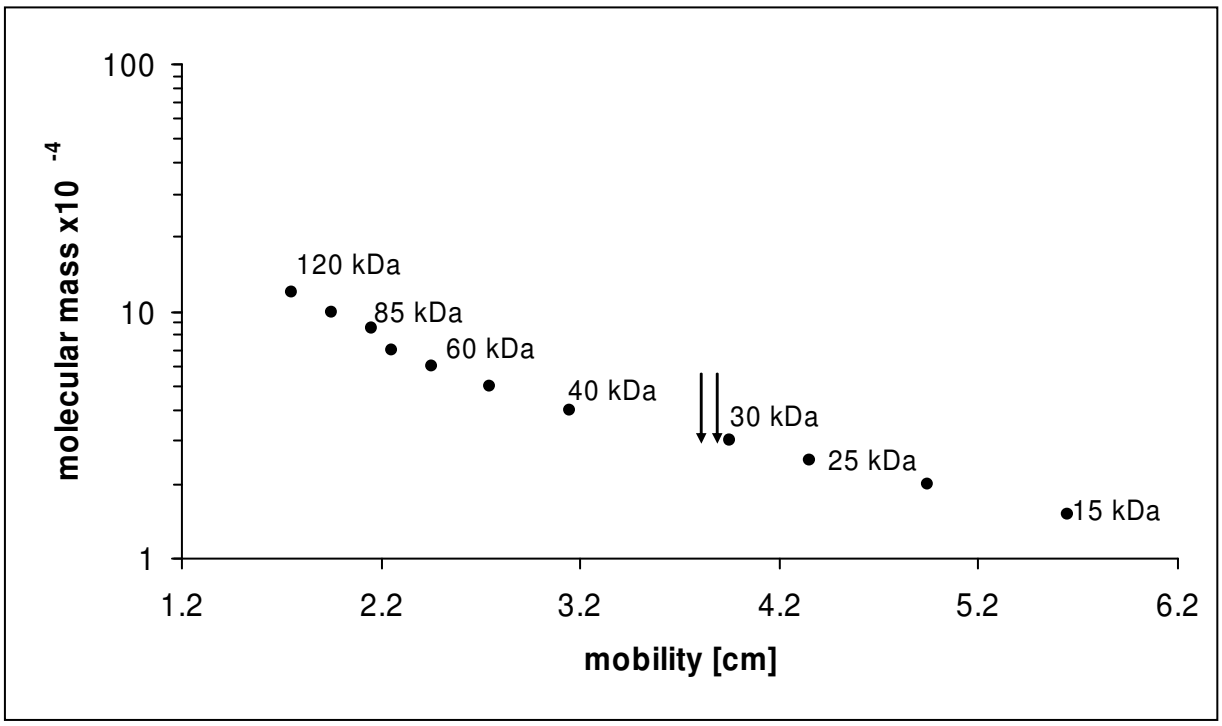


Fig. 4-38: Determination by SDS-PAGE of the molecular masses of the two polypeptides in fraction 12 after gel filtration.

⇓⇓ **Positions of the two protein bands**

4.3.5.2 Determination of the native molecular mass by gel filtration

The purified prenyltransferase was eluted in fraction 12 (Fig.4-35). This position corresponds, on the basis of the calibration curve (Fig. 4-33), to a relative molecular mass of ~ 63000. This value was confirmed by using ammonium sulphate-precipitated protein (30-65% saturation) for gel filtration on a calibrated Sephacryl 200 HR column. The prenyltransferase activity was detected at Ve/Vo 2.03, which also indicates a relative molecular mass of ~ 63000.Thus; the

purified prenyltransferase appeared to be a heterodimer of the two subunits that were detected in SDS-PAGE.

4.3.6 Two-dimensional (2-D) electrophoresis

The combination of isoelectric focusing (IEF) and SDS-PAGE is regarded to be the most powerful separation method for resolving complex mixtures of proteins into distinct spots in a gel. Gel plugs of these spots can be removed and further analyzed by mass spectrometry to determine the identity of individual proteins.

4.3.6.1 First-Dimension Isoelectric Focusing (IEF)

Approximately 10 µg of the purified protein were solubilized in rehydration buffer and loaded onto an IPG strip (3.6.1). A pH 3–10 IPG strip allows detection of a broad spectrum of protein spots.

4.3.6.2 Second-dimension SDS-PAGE

After separation in the first dimension, the proteins were further resolved according to their molecular masses using a 12% gel (3.6.2)

4.3.6.3 Detection and analysis of 2-D resolved protein spots

Staining with Coomassie-Blue visualized four protein spots (Fig. 4-39). One of them was stained intensively (spot 2). The four protein spots were excised from the 2 D-gel and subjected to enzymatic digestion (4.3.7), leading to a series of peptides for sequencing.

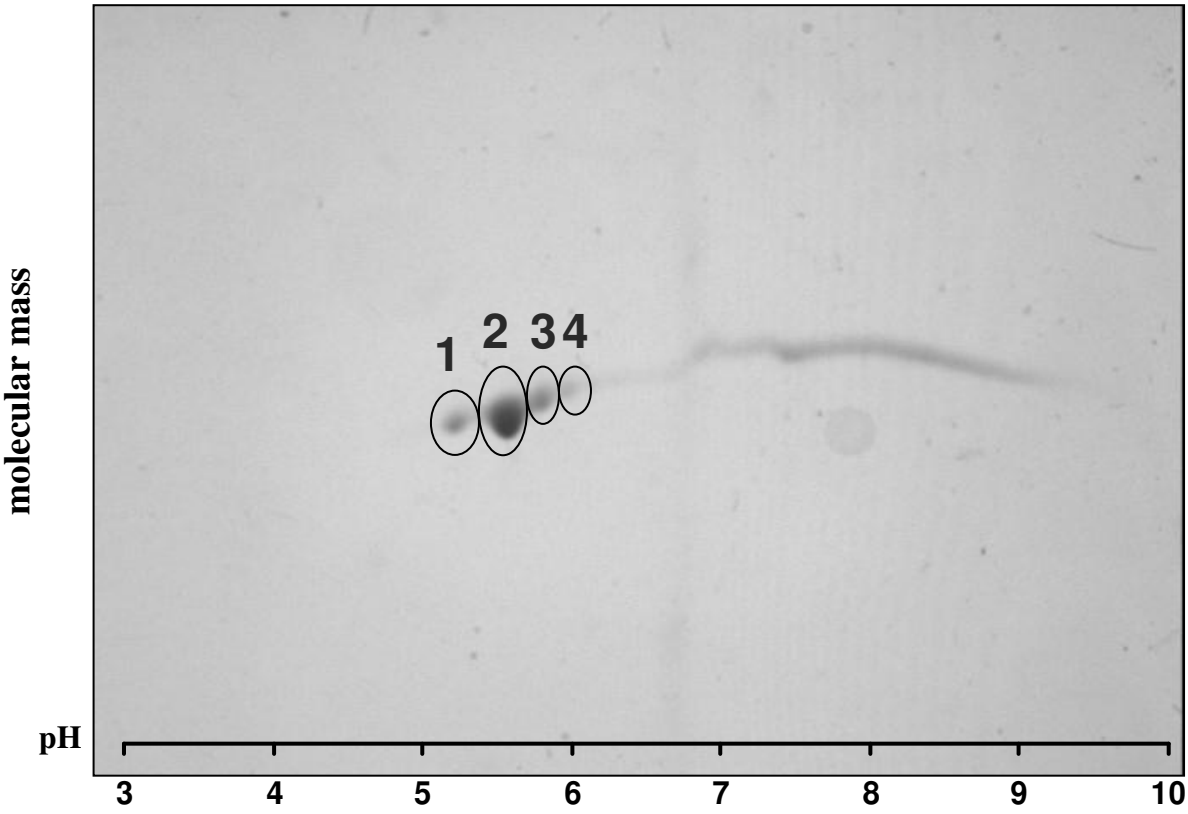


Fig. 4-39: Two-dimensional electrophoresis of the purified protein (Coomassie-Blue-stained)

4.3.7 Partial sequencing of the separated polypeptides

The enzymatic digestion with trypsin and the subsequent partial sequencing of the resulting peptides by mass spectrometric analysis was carried out by Dr. Nimtz at the Gesellschaft für Biotechnologische Forschung (GBF) in Braunschweig.

Mass spectrometry (MS) is an important and emerging method for the identification of gel-separated proteins and makes use of the rapidly growing sequence databases. Generally, there are two MS methods that can be used to identify proteins: matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) and tandem mass spectrometry (MS/MS). Both methods work best when trypsin digestion is used to break up each protein into multiple peptide fragments.

The four protein spots were excised from the Coomassie-Blue-stained 2-D gel and subjected to tryptic digestion to yield a mixture of peptide fragments. The larger peptide fragments were analysed by mass spectrometry using electrospray ionization tandem-MS (ESI-MS/MS). The resulting tryptic peptide sequence information served for database searching using the Fasta database search program Uniprot (http://www.ebi.ac.uk/fasta33/). The Fasta program can be very specific when identifying long regions of low similarity especially for highly diverged sequences.

This search consistently detected significant homology between the larger peptide fragments and the elongation factor 2, but no homology was found with prenyltransferases and terpene synthases. Obviously, the major portion of the purified protein was elongation factor 2. An additional protein spot possibly containing the prenyltransferase was unfortunately not detectable in 2D-electrophoresis.

5. Discussion

Hyperforin is an important constituent of St. John's wort preparations. While the pharmacological activities of this compound have intensely been studied, little is known about the biosynthesis of hyperforin. Recently, the formation of hyperforin and its homologue adhyperforin has been detected in *H. calycinum* cell suspension cultures (Klingauf et al., 2005). Cell cultures of other *Hypericum* species (*H. perforatum, H. androsaemum, H. gnidioides*) failed to accumulate hyperforins. Thus, cell cultures of *H. calycinum* turned out to be a valuable *in vitro* system for studying the biosynthesis of hyperforins (Klingauf et al., 2005). Contrary to intact *H. perforatum* plants which accumulate hyperforin, cell cultures of *H. calycinum* contain mainly the homologue adhyperforin. The antidepressant activity of adhyperforin resembles that of hyperforin ((Maisenbacher and Kovar, 1992; Jensen et al., 2001). So far, hyperforins have not been detected in differentiated *H. calycinum* plants, although several dearomatized polyprenylated acylphloroglucinols have been isolated from this species (Decosterd et al., 1998; Gronquist et al., 2001). One of these constituents showed antimalarial activity in an *in vitro* test system (Decosterd et al., 1991). Where the lipophilic compounds accumulate in differentiated plants and cultured cells of *Hypericum* species is still open. In mice, alcoholic extracts from *H. calycinum* exhibited antidepressant activity which compared to that of extracts from *H. perforatum* (Öztürk, 1997). In the same study, *H. hyssopifolium ssp.* had no antidepressant activity.

In flowers of *H. calycinum*, the ecological function of polyprenylated acylphloroglucinol and benzophenone derivatives has recently been studied (Gronquist et al., 2001). These compounds accumulate in those parts of petals which are exposed to the outside in the unopened bud. This is the first time that constituents other than flavonoids were found to serve as floral UV pigments. Interestingly, the polyprenylated compounds also accumulate to a high extent in anthers and the ovarian wall and provide protection against herbivores. Thus, they fulfill both attractive and defensive functions in *H. calycinum* flowers.

The skeleton of hyperforin is the phloroglucinol derivative phlorisobutyrophenone, the formation of which is catalyzed by isobutyrophenone synthase (BUS). This enzyme catalyzes the sequential condensation of one molecule of isobutyryl-CoA with three molecules of malonyl-CoA to form the hyperforin nucleus (Klingauf et al., 2005; Fig. 5-1). An increase in BUS activity preceded the formation of hyperforins during cell culture growth.

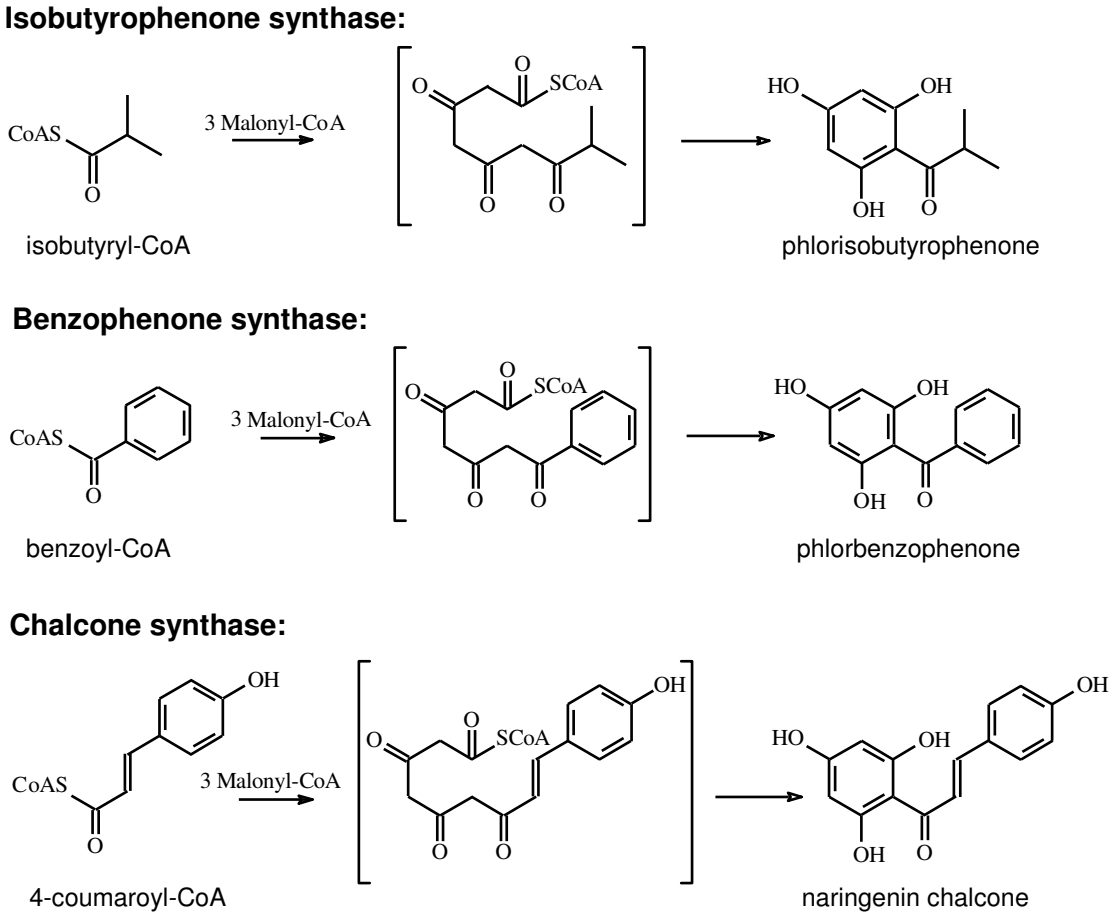


Fig. 5-1: Reactions of three type III polyketide synthases from *H. calycinum* cell cultures

That a polyketide mechanism underlies the formation of the hyperforin skeleton, was confirmed by a retrobiosynthetic NMR study (Adam et al., 2002). Cultured *H. calycinum* cells contained, besides BUS, chalcone synthase (CHS) and benzophenone synthase (BPS) activities. These enzymes were separated by anion exchange chromatography. While the preferential starter substrate of BPS is benzoyl-CoA, leading to the formation of phlorbenzophenone, CHS prefers 4-coumaroyl-CoA, resulting in the formation of naringenin chalcone. Both products can undergo intramolecular cyclization to give xanthones and flavonoids, respectively. BUS, BPS and CHS belong to the superfamily of type III polyketide synthases. These enzymes generate a diverse array of secondary metabolites by condensing multiple acetyl units derived from malonyl-CoA to specific starter molecules (Jez et al., 2002). The homodimeric enzymes orchestrate a series of acyltransferase, decarboxylation, condensation, cyclization, and aromatization reactions at two functionally independent active sites.

The structure of the active site dictates starter substrate preference, polyketide chain-length, and regio-specificity of polyketide cyclization. These strategies have increased considerably the functional diversity of type III PKSs.

In hyperforin biosynthesis, phlorisobutyrophenone undergoes a series of prenylation reactions. A proposed biosynthetic pathway (Adam et al., 2002) is depicted in Fig. 5-2. The unsubstituted acylphloroglucinol precursor requires a triple electrophilic substitution of the aromatic nucleus involving two DMAPP units and one geranyl pyrophosphate as well as a ring closure triggered by electrophilic attack of a third DMAPP unit. The sequence of these steps remains to a large extent unidentified. Mechanistic considerations require the cyclization to be preceded by quaternization of the carbon atom to which the geranyl chain is appended. Subsequent quaternization of a preformed bicyclic intermediate would involve the participation of an unduely strained bridgehead enolate ion. One of the remaining possibilities satisfying this restriction and substantiating an earlier suggestion (Bystrov et al., 1975) is illustrated in Fig. 5-2.

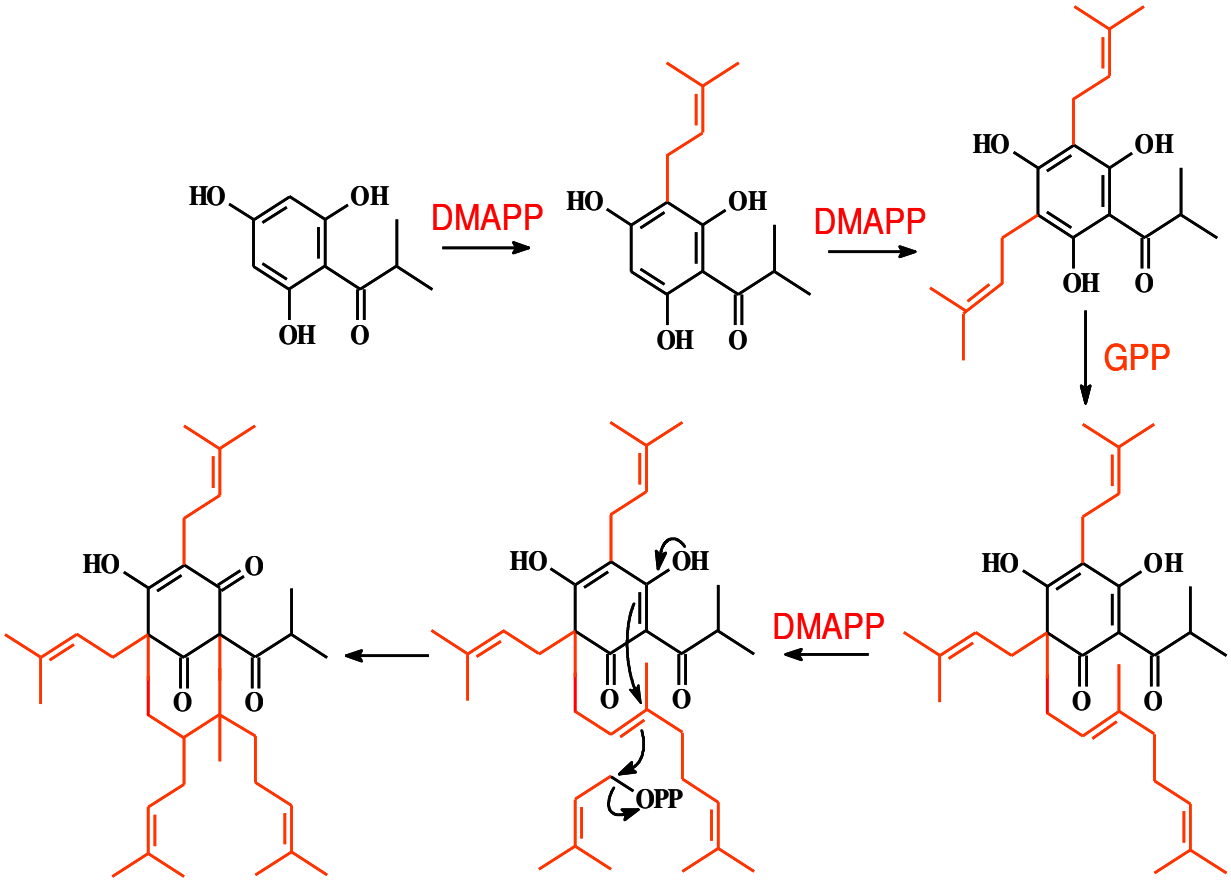


Fig. 5-2: Hypothetical pathway of hyperforin biosynthesis

The five isoprenoid moieties which participate in hyperforin biosynthesis are derived predominantly (>98%) via the non-mevalonate pathway, as shown by a retrobiosynthetic NMR study (Adam et al., 2002). Involved are a series of enzyme-catalyzed reactions summarized in Fig. 5-3. The initial step of this route is the formation of 1-deoxy-D-xylulose 5-phosphate **3** (DXP) by condensation of glyceraldehyde 3-phosphate **2** and (hydroxyethyl) thiamine resulting from the decarboxylation of pyruvate **1.** This reaction is catalyzed by the thiamine diphosphate-dependent DXP synthase (DXS). The second enzyme of this biosynthetic pathway, the DXP reductoisomerase (DXR), catalyzes the transformation of DXP into methylerythritol MEP **5**. The third step is conversion of MEP **5** into 4-diphosphocytidyl-2-*C*-methyl-D-erythritol **6** by the 4-diphosphocytidyl-2-C-methyl-D-erythritol-synthase. Subsequent phosphorylation of the C-2 hydroxyl group of **6** yields **7**. 4-Diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphat **7** is converted by the 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase to 2C-Methyl-D-erythritol-2,4-cyclodiphosphat **8**. (E)-4-Hydroxy-3-methylbut-2-enyl diphosphate **9** is a transient intermediate, directly derived from cMEPP **8**, which is subsequently dehydroxylated to yield IPP/DMAPP (**10/11**). Thus, the deoxyxylulose phosphate pathway in higher plants appears to be completely elucidated at the level of intermediates. Recent studies have shown a wide variety of plant terpenoids to be formed by the non-mevalonate pathway (Dubey et al., 2003), such as isoprene from *Chelidonium majus* (Zeidler et al., 1997), thymol from *Thymus vulgaris* (Eisenreich et al., 1997), β-carotene from *Liriodendron tulipifera* (Sagner et al., 1998), cineole from *Eucalyptus globulus* (Rieder et al., 2000) and borneol from *Conocephalum conicum* (Theil and Adam., 2002). The plastids have long been accepted as a major subcellular site of isoprenoid metabolism this finding is underscored by the recent discovery that these organelles possess a DXP pathway for the production of the universal isoprenoid precursor IPP (Eisenreich et al., 1998). In the case of prenylated aromatics, it has been reported that the dimethylallyl group of several aromatic compounds (Goese et al., 1999; Stanjek et al., 1999; Asada et al., 2000) and the geranyl group of cannabinoids (Fellermeier et al., 2001) are derived from the DXP pathway. Furthermore, prenylation of isoflavonoids in French bean (*Phaseolus vulgaris*) and soybean (*Glycine max*; Biggs et al., 1990) and of furanocoumarins in *Ruta graveolens* (Dhillon and Brown, 1976) occurrs in the plastids. In contrast, in cultured *Lithospermum erythrorhizon* cells, GPP supplied by the mevalonate pathway (Li et al., 1998) in the ER/cytosol (Sommer et al., 1995) was utilized for the prenylation of *p*-hydroxybenzoic acid, which was associated with the ER (Yamaga et al., 1993).

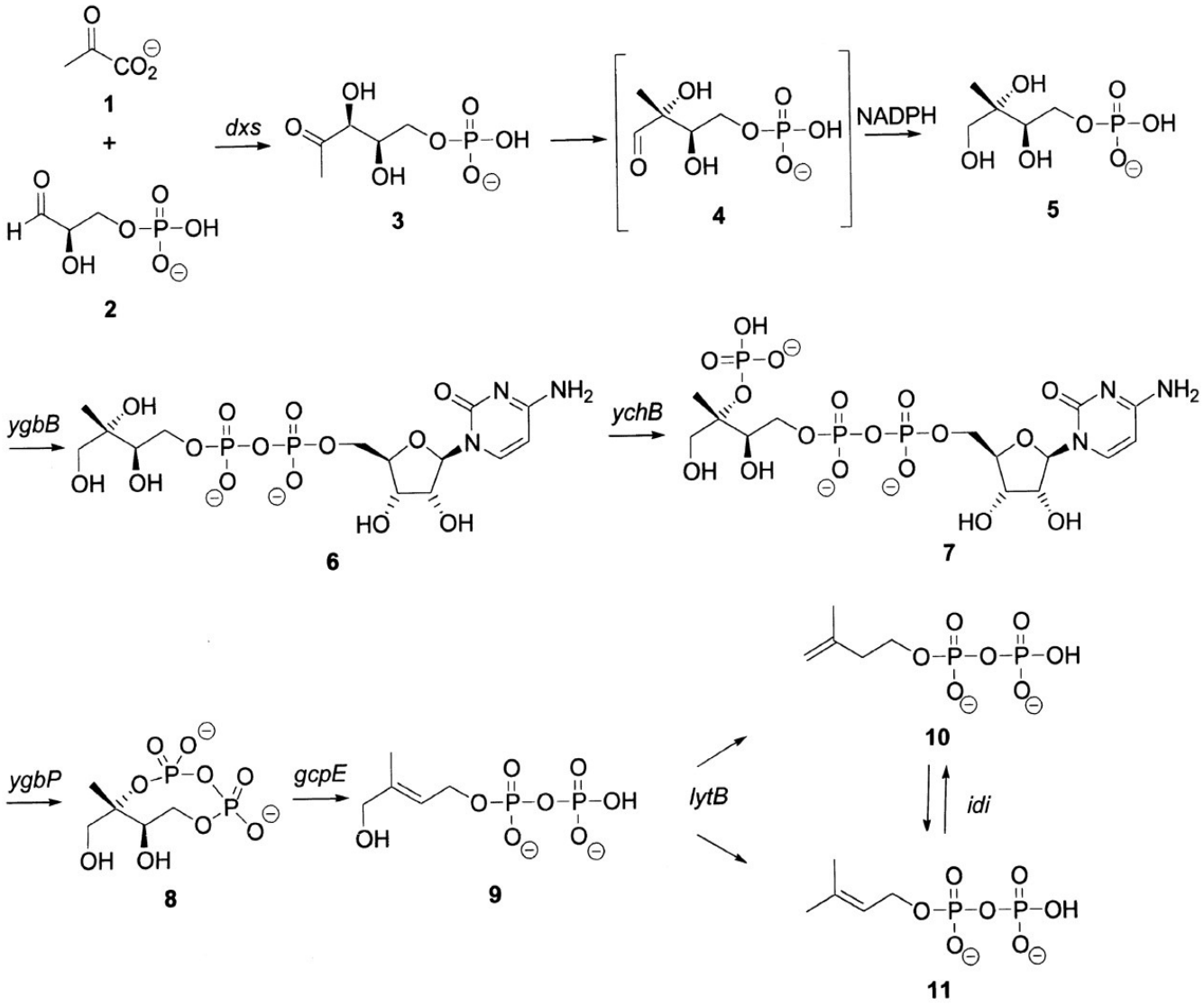
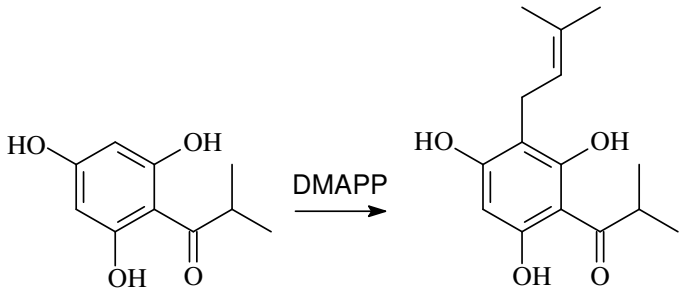


Fig. 5-3: Deoxyxylulose phosphate (non-mevalonate) pathway leading to the formation of isopentenyl diphosphate (10) and dimethylallyl diphosphate (11)

The present work reports detection, characterization and partial purification of the dimethylallyltransferase that is responsible for the first prenylation step in hyperforin biosynthesis. The enzyme catalyses the conversion of phlorisobutyrophenone to dimethylallylphlorisobutyrophenone in the presence of DMAPP and Fe^{2+} (Fig. 5-4). The enzymatic product was unequivocally identified as dimethylallylphlorisobutyrophenone by ^{1}H NMR and MS analyses.



Phlorisobutyrophenone Dimethylallylphlorisobutyrophenone

Fig. 5-4: Prenylation of the hyperforin nucleus by phlorisobutyrophenone dimethylallyltransferase

Hyperforin accumulation between day 3 and 6 of cell culture growth was preceded by an increase in dimethylallyltransferase activity, strongly indicating that this enzyme is involved in hyperforin biosynthesis. Other pathways involving prenylation reactions in *Hypericum* species lead to the formation of polyprenylated benzophenone derivatives. Some previously characterized prenyltransferases are *p*-hydroxybenzoate polyprenyltransferase involved in ubiquinone biosynthesis (Thomas and Threlfall, 1973), homogentisate polyprenyltransferase participating in the biosynthesis of plastoquinone and tocopherol (Pennock, 1985; Marshall et al., 1985) and 1, 4-dihydroxynaphthoate polyprenyltransferase leading to phylloquinone formation (Schultz et al., 1981).

Prenyltransferases catalyze the electrophilic alkylation of electron-rich acceptor substrates by the hydrocarbon moiety of allylic isoprenoid diphosphates (Poulter and Rilling, 1978; Poulter et al., 1979). Some of the more common acceptors are carbon-carbon double bonds (synthesis of isoprenoid chains; Szkopiñska, 2000), aromatic rings (e.g. synthesis of respiratory quinones and vitamins E; Ashby and Edwards, 1990), amino groups (e.g modification of tRNAs; Caillet and Droogmans, 1988) and sulfhydryl moieties (e.g modification of proteins such as ras-farnesyltransferase and anti-proliferation target; Stöber et al., 1997, Mayer et al., 1993). In these prenyl side chain conjugation reactions, the adequate length of the prenyl diphosphate which is biosynthesized before-hand by prenyl diphosphate elongation enzymes is thought to be directly transferred to the prenyl acceptor (Fellermeier and Zenk, 1998; Mühlenweg et al., 1998; Liang et al., 2002). The products of prenyl transfer reactions are ultimately converted into over 30000 naturally occurring isoprenoid compounds and it is well known that many prenyl side chain-conjugated compounds have important biological functions, such as defence

and attraction (Barron and Ibrahim, 1996). Isoprenoid biosynthesis is essential in all organisms. In *Escherichia coli*, for instance, the isoprenoids ubiquinone and bactoprenol are required for respiration and cell wall biosynthesis, respectively. The aromatic polyprenyltransferase encoded by ubiA is involved in the biosynthesis of prenylated quinones (Shineberg and Yong, 1976). The enzyme transfers diphosphorylated acyclic oligoprenyl moieties (diphosphorylated terpene alcohols) to the meta-position of 4-hydroxybenzoic acid (Wessjohann and Sontag, 1996; Wessjohann et al., 1999; Wessjohann and Sontag, 1998). Prenyltransferases also participate in the biosynthesis of flavonoids (Yamamoto et al., 2000), coumarins (Hamerski et al., 1990), cannabinoids (Fellermeier and Zenk, 1998), hop bitter acids (Zuurbier et al., 1998), and shikonin (Yazaki et al., 2002). The shikonin-related enzyme was the first prenyltransferase to be cloned from a plant source, namely cell cultures of *Lithospermum erythrorhizon.* The enzyme catalyzes the transfer of a geranyl residue to 4-hydroxybenzoic acid.

The dimethylallyltransferase which is responsible for the first prenylation step in hyperforin biosynthesis is a so-called aromatic prenyltransferase which catalyzes the formation of a carbon-carbon bond between an aromatic nucleus and a prenyl group. The majority of the aromatic prenyltransferases are integral membrane proteins (Dhillon and Brown, 1976; Schröder et al., 1979; Biggs et al., 1987; Hamerski et al., 1990; Welle and Grisebach, 1991; Laflamme et al., 1993; Yamamoto et al., 1997, 2000; Mühlenweg et al., 1998). Their catalytic centers include a typical prenyl disphophate binding site: (N/D)DXXD. Examples of this enzyme family are the prenyltransferases involved in the biosynthesis of the primary metabolites ubiquinone (Turunen et al., 2004), menaquinone (Suvarna et al., 1998), tocopherol (Schledz et al., 2001) and plastoquinone (Collakova and DellaPenna, 2001). The same binding motif is also present in the chain-elongating trans-prenyltransferases such as geranyl diphosphate synthase, farnesyl diphosphate synthase, and geranylgeranyl diphosphate synthase (Wang and Ohnuma, 1999; Bohlmann et al., 1998; Liang et al., 2002; Sacchettini and Poulter, 1997). The geranyltransferase cloned from *L. erythrorhizon* also belongs to this group (Yazaki et al., 2002).

Another class of aromatic prenyltransferases includes soluble enzymes which are, in addition, active in a metal-free buffer containing EDTA. An example is dimethylallyltryptophan synthase (DMATS) which catalyzes the prenylation of tryptophane at position C-4 of the indole nucleus in ergot alkaloid biosynthesis in the fungus *Claviceps purpurea* (Cress et al.,

1981; Lee et al., 1976; Tsai et al., 1995; Tudzynski et al., 1999). Further soluble prenyltransferases that use aromatic substrates and are active in the absence of metal ions and lack the (N/D)DXXD motif are CloQ which is involved in the biosynthesis of clorobiocin from *Streptomyces roseochromogenes* (Pojer et al., 2003), and LtxC which participates in the biosynthesis of lyngbyatoxins from *Lyngbya majuscula* (Edwards and Gerwick., 2004). Very recently, an orthologue of DMATS (FgaPT2) was identified in the genome sequence of *Aspergillus fumigatus* (Unsöld and Li, 2005). Interestingly, DMATS, CloQ and LtxC share no a ppreciable sequence similarity, despite their similar biochemical properties.

The dimethylallyltransferase from cultured *H. calycinum* cells differs from the members of the above-mentioned classes of prenyltransferases in that it is soluble and its activity dependent on a divalent cation. It is the first plant prenyltransferase to prefer Fe^{2+}. Other enzymes are most active with Mg^{2+} (Hamerski et al., 1990; Yamamoto et al., 1997; Fellermeier and Zenk, 1998; Mühlenweg et al., 1998; Yamamoto et al., 2000; Tholl et al., 2001) or Mn^{2+} (Dhillon and Brown, 1976; Biggs et al., 1987; Welle and Grisebach, 1991; Laflamme et al., 1993). Purified dimethylallyl:tRNA dimethylallyltransferase from *Escherichia coli* which is involved in adenosine biosynthesis prefers Mn^{2+} and Fe^{2+} as cofactor (Bartz and Söll, 1972). In contrast, many protein prenyltransferases contain a tightly bound zinc atom (Harris et al., 2002). The dimethylallyltransferase from cultured *H. calycinum* cells exhibited with Zn^{2+} only 41% of the activity obtained in the presence of Fe^{2+}. The maximum specific activity of this enzyme in crude extracts was 3.6 µkat /kg. This value is low, as compared to the activity of kaempferol 8-dimethylallyl transferase in *Epimedium diphyllum* cell cultures (23.3 µkat /kg; Yamamoto et al., 1997), prenyltansferase in hop bitter acids (38 µkat /kg; Zuurbier et al., 1998), and 4-hydroxybenzoic acid geranyltransferase from shikonin-producing *L. erythrorhizon* cell cultures (68.4 µkat /kg; Heide and Tabata, 1987). In contrast to previously reported prenyltransferase reactions for which the optimum pH was around 7.5 (Dhillon and Brown, 1976; Schröder et al., 1979; Biggs et al., 1987; Hamerski and Matern, 1988; Welle and Grisebach, 1991; Laflamme et al., 1993; Fellermeier and Zenk, 1998; Mühlenweg et al., 1998; Tholl et al., 2001), the prenyltransferase from cultured *H. calycinum* cells showed a broad pH optimum from 6.5 to 9. A similarly broad pH range was observed with the prenyltransferase from *Epimedium diphyllum* cell cultures (Yamamoto et al., 1997).

Some prenyltransferases previously characterized are remarkably similar to the dimethylallyltransferase from cultured *H. calycinum* cells, i.e. they are soluble and require a divalent metal ion. These enzymes are involved in the biosynthesis of bitter acids in hop (*Humulus lupulus*; Zuurbier et al., 1998) and of cannabinoids in hemp (*Cannabis sativa*; Fellermeier and Zenk, 1998). In glandular hairs of hop cones, the enzyme activity catalyzes the stepwise prenylation of acylphloroglucinols to yield deoxyhumulone and deoxycohumulone which are the direct precursors of the hop bitter acids. In leaves of Indian hemp, the enzyme catalyzes the alkylation of olivetolic acid with GPP. It will be interesting to study the evolutionary relationship between these enzymes which include the dimethylallyltransferase from *H. calycinum* cell cultures and the members of the two other classes of prenyltransferases.

The K_m values of the *H. calycinum* enzyme for DMAPP and phlorisobutyrophenone were in the range of Michaelis constants found for other plant aromatic prenyltransferases (Yamamoto et al., 1997). Dimethylallyltransferase links a dimethylallyl group to phlorisobutyrophenone, with the maximum reaction rate being observed at 2 to 8 mM DMAPP. Higher concentrations inhibited the enzyme (Fig. 4-17), as previously found with the geranyltransferase in cell-free extracts from cultured *Lithospermum erythrorhizon* cells, which catalyzes the geranylation of 4-hydroxybenzoic acid (Heide and Tabata, 1987). Welle and Grisebach (1991) revealed that dimethylallyl diphosphate:trihydroxypterocarpan dimethylallyltransferase from *Glycine max* was competitively inhibited by IPP. Inhibition of the *H. calycinum* enzyme is thought to be also due to IPP formation from DMAPP by IPP isomerase present in cell-free extracts. Conversely, product formation occurred in the presence of IPP, which was likewise attributed to IPP isomerase activity in crude extracts. Dimethylallyltransferase accepted only DMAPP as prenyl donor. Geranyl pyrophosphate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate were not transferred to phlorisobutyrophenone (Table 4-2), indicating that the dimethylallyltransferase strictly recognizes the chain length of the prenyl donor. Similarly, dimethylallyltransferase showed high specificity for phlorisobutyrophenone which is the physiological acceptor, i.e. the acylphloroglucinol moiety of hyperforin. While the structurally closely related substrate phloracetophenone was also efficiently converted, phloroglucinol was not.

Recently, a new branch point in the biosynthesis of hyperforins was detected (Fig. 5-5; Charchoglyan et al., 2006). Shoot cultures of *H. perforatum* were found to contain hyperforin and three related polyprenylated acylphloroglucinol derivatives. The accumulation of these compounds was coupled to shoot regeneration, with secohyperforin being the prominent constituent in morphogenic cultures. In multiple shoot cultures, the ratio of hyperforin to secohyperforin was strongly influenced by the phytohormones N6- benzylaminopurine (BAP) and naphthalene-1-acetic acid (NAA). While increasing concentrations of BAP stimulated the formation of hyperforin, increasing concentrations of NAA elevated the level of secohyperforin. Hyperforin and secohyperforin are likely to result from a branch point in the biosynthetic pathway. The diprenylated acylphloroglucinol intermediate is proposed to be linked to either a dimethylallyl or a geranyl residue. Metabolic channeling at the branch point might be due to differentially regulated prenyltransferases exhibiting specificity for DMAPP or GPP.

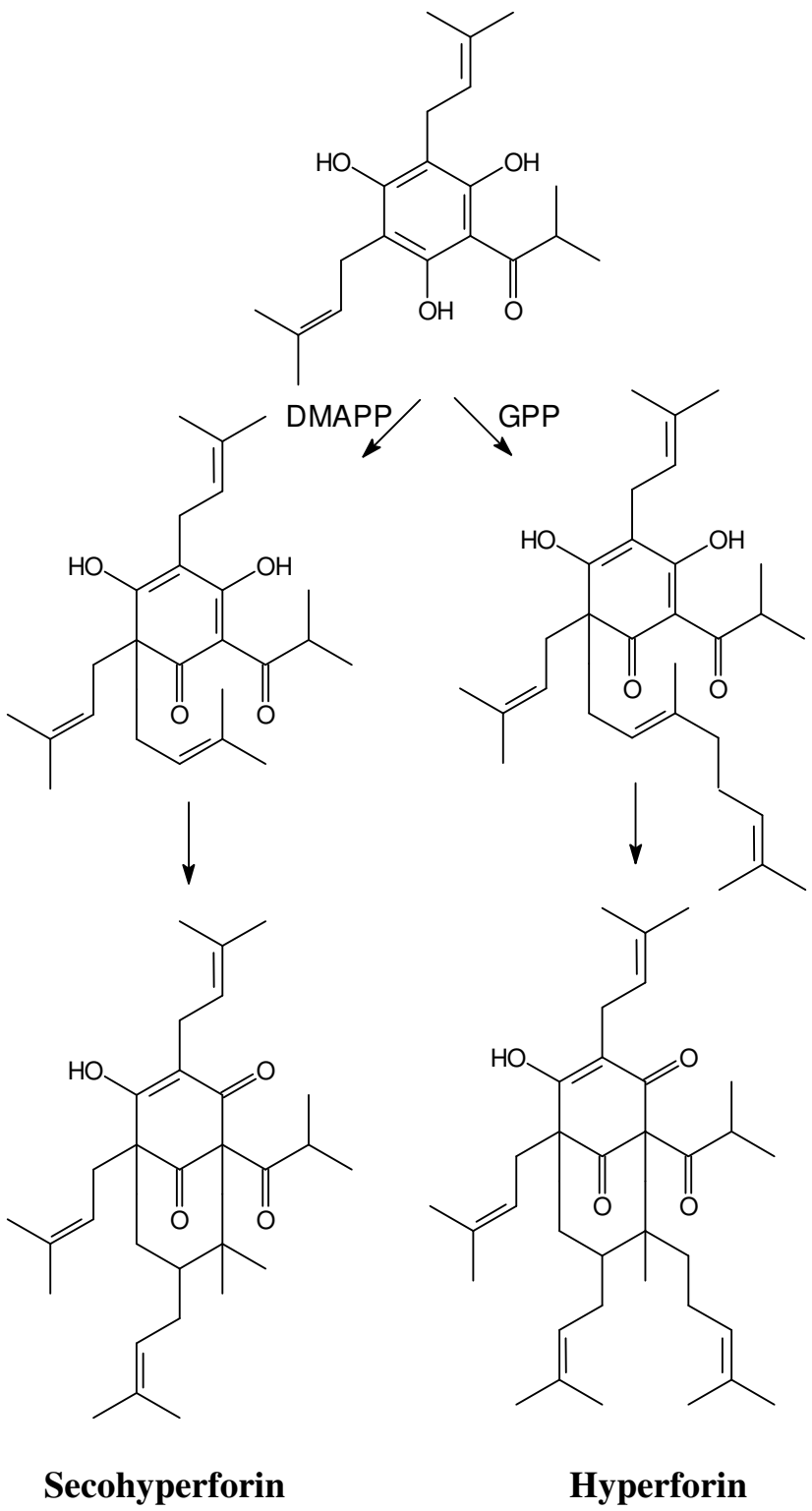


Fig. 5-5: Proposed branch point in the biosynthetic pathway of hyperforins

Hypericum species contain, besides prenylated acylphloroglucinols such as hyperforin, polyprenylated benzophenone derivatives (Fig. 5-6). The skeleton of these constituents is formed by BPS catalyzing the iterative condensation of benzoyl-CoA with three molecules of

malonyl-CoA to give phlorbenzophenone. In *H. androsaemum* cell cultures, 3'-hydroxylation of this intermediate to yield 2,3',4,6-tetrahydroxybenzophenone is catalyzed by a cytochrome P450 monooxygenase (Schmidt and Beerhues, 1997). In cultured *Centaurium erythraea* cells, BPS prefers 3-hydroxybenzoyl-CoA as starter substrate, giving immediately the tetrahydroxylated metabolite (Beerhues, 996). 3-Hydroxybenzoate: CoA ligase was separated from 4-coumarate: CoA ligase and purified to homogeneity (Barillas and Beerhues., 2000). Simple benzophenones can undergo stepwise prenylation and cyclization reactions to give bridged polycyclic compounds such as sampsoniones and garcinols. Some of these complex molecules with caged skeletons exhibit interesting pharmacological properties, e.g. cytotoxic, antibacterial, and HIV-inhibitory activities (Balasubramanyam et al., 2004; Hu and Sim., 1999; Matsumoto et al., 2003). Their mechanisms of action and target structures are under study.

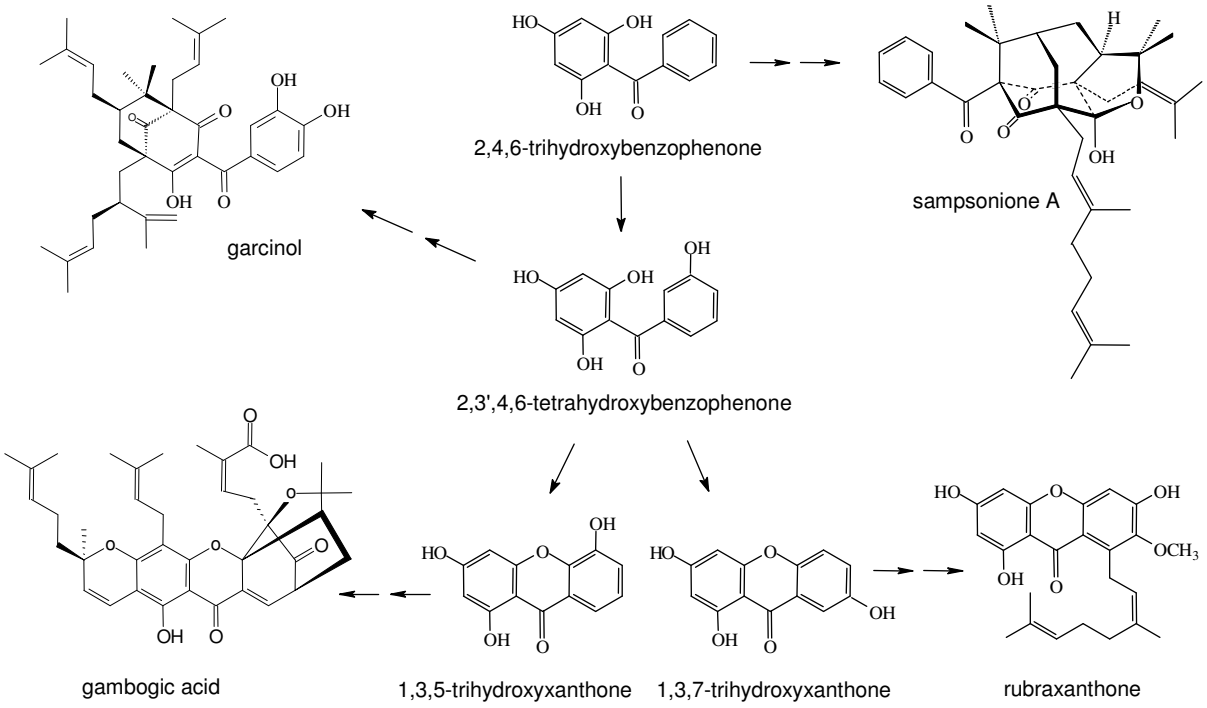


Fig. 5-6: Polyprenylated benzophenone and xanthone derivatives

2,3',4,6-Tetrahydroxybenzophenone is also the precursor of xanthone biosynthesis (Fig. 5-6). It undergoes regioselective oxidative phenol coupling reactions which occur either *ortho* or *para* to the 3'-hydroxy group and yield 1,3,5- and 1,3,7-trihydroxyxanthones, respectively (Peters et al., 1998). These intramolecular cyclizations are catalyzed by cytochrome P450 enzymes. The two isomeric products are the precursors of the majority of plant xanthones. This class of secondary metabolites likewise includes prenylated molecules with high pharmaceutical potential. Pronounced activity against methicillin-resistant strains of *Staphylococcus aureus* was observed with rubraxanthone, the efficiency of which was comparable to that of the antibiotic vancomycin (Iinuma et al., 1996). An example of a bridged polycyclic xanthone is gambogic acid which induces apoptosis independent of cell cycle by a novel mechanism of caspase activation (Zhang et al., 2004).

Since most aromatic prenyltransferases are membrane-bound, their purification is difficult. Little progress towards solubilization and purification of plant prenyltransferases was described. Successful solubilization procedures were reported for the isoflavonoid prenyltransferase in soybean (Welle and Grisebach, 1991) and a peripheral membrane protein of rutacridone biosynthesis in *Ruta graveolens* cell cultures (Maier et al., 1993). Partial purification was achieved with the 4-hydroxybenzoate geranyltransferase from *L. erythrorhizon* (Mühlenweg et al., 1998). In contract, the dimethylallyltransferase from *H. calycinum* cell cultures is a soluble enzyme as has been shown in Tab. 4-3. This provides a good basis for a successful purification strategy. However, the activity of the dimethylallyltransferase was comparatively low and it could not be increased by elicitor treatment (Klingauf et al., 2005). A purification scheme for this enzyme utilizing differential anionic, hydrophobic, adsorptive and size properties of proteins was established. Hydrophobic interaction chromatography turned out to be an efficient purification step because a large portion of foreign protein was removed, leading to a considerable increase in the purification factor. Dimethylallyltransferase was eluted from the HIC column at high concentrations of ammonium sulphate, suggesting that the enzyme does not belong to the hydrophobic proteins.

The purification scheme, as outlined in Fig. 4-34, led to a considerable increase in prenyltransferase purity (Tab. 4-4) and finally allowed association of protein bands in SDS–PAGE with dimethylallyltransferase activity. In order to obtain a highly purified dimethylallyltransferase preparation, only fractions exhibiting high enzyme activity were pooled and subjected to the next purification step. This, however, resulted in a relatively low

recovery (0.29%) and a poor purification factor (65). Previously, a low yield was also observed with the purified 4-hydroxybenzoate geranyltransferase from *L. erythrorhizon* cell cultures (Mühlenweg et al., 1998). Prenyltransferases, in addition, appear to be relatively unstable proteins during the purification procedure. The six step-purification process provided an apparently homogeneous preparation of dimethylallyltransferase protein, as determined by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Two closely-migrating protein bands with molecular masses of about 31 and 31.5 kDa were detected. The native molecular mass of the prenyltransferase, as estimated by gel-filtration chromatography, was about 63 kDa. These results suggested that the purified enzyme was active as a heterodimer. A prenyltransferase from the fungus *Aspergillus fumigatus*, encoded by the gene *fgaPT1*, has recently also been found to be active as a dimer (Unsöld and Li, 2006).

The final separation was achieved by two-dimensional gel electrophoresis, leading to the detection of four spots. These polypeptides were amenable to identification by in-gel trypsin digestion, followed by ESI–MS/MS sequence analysis of the resulting tryptic peptides. So far, only few sequences for "aromatic" prenyltransferases from plants are available. Unfortunately, none of the tryptic peptides exhibited amino acid sequence similarity to known prenyltransferases and terpene synthases. In contrast, all four polypeptides separated by 2D electrophoresis turned out to be isoforms of the elongation factor 2. Elongation factors of protein synthesis constitute one of the major families of cellular GTP-binding proteins, also called GTPases. Members of the GTPase superfamily are characterized by conserved structural motifs and their ability to alternate between specific conformations by binding either GTP or GDP. The alternate conformations associated with the binding and hydrolysis of GTP allows the GTPases to function as “molecular switches” in the specific activation of other cellular components. In the elongation phase of protein synthesis, the translocation of the ribosome along the messenger RNA requires an elongation factor (EF-G in prokaryotes and EF-2 in eukaryotes) and is accompanied by the hydrolysis of GTP. It is presumed that the translocation process is driven by a change in the conformation of the EF-2 GTP-binding domain associated with GTP hydrolysis.

In conclusion, the purification procedure that was established in this work turned out to be in principal suitable for the isolation of the soluble prenyltransferase from *H. calycinum* cell cultures. Enzyme activity could be profiled during the six-step procedure. In the future, the individual chromatographic steps will be further improved and the amount of cell-free extract

as starting material will be increased. This strategy will hopefully provide purified dimethylallyltransferase protein, from which partial sequences can be derived as a basis for subsequent cDNA cloning.

6. SUMMARY

- St. John's wort (*Hypericum perforatum* L., Clusiaceae) has been used as a medicinal herb for over 2000 years. Application of its extracts for treating depression has undergone rigorous scientific investigation. The major antidepressant constituent appears to be hyperforin. This polyprenylated phloroglucinol derivative exhibits a broad-band inhibitory effect on the neuronal reuptake of serotonin, noradrenaline, dopamine, gamma-aminobutyric acid (GABA), and L-glutamate. It is both a structurally and functionally new antidepressant.

- The enzyme which catalyzes the first prenylation step in hyperforin biosynthesis was detected in cell cultures of *H. calycinum* grown in BDS medium in the dark. Thus, these cell cultures turned out to be a valuable *in vitro* system for studying hyperforin biosynthesis.

- Dimethylallyl diphosphate (DMAPP) was chemically synthesized as a donor substrate. It was incubated with cell-free extracts from *H. calycinum* cell cultures in the presence of a divalent cation and phlorisobutyrophenone as the acceptor substrate. These enzyme assays resulted in the formation of dimethylallylphlorisobutyrophenone, as identified by HPLC and GC-MS in comparison with a chemically synthesized reference compound.

- The responsible enzyme showed a broad pH optimum from 6.5 to 9 and a temperature optimum at 38°C. The enzyme reaction was linear with time up to 30 min and with the protein amount up to 80 µg in the assay.

- Dimethylallyltransferase activity was strictly dependent on a divalent cation, with Fe^{2+} being the most efficient cofactor. Lower activities were observed with Mg^{2+}, Zn^{2+}, and Cu^{2+}. The optimum ferrous concentration was 10 mM. The preference for ferrous was not due to its reducing properties because experiments in the presence and the absence of 10 mM ascorbic acid gave the same result.

- The transferase used DMAPP as prenyl donor. Product formation also occurred in the presence of IPP, which, however, was attributed to IPP isomerase activity in the crude extract. No enzyme activity was observed with GPP, FPP, and GGPP as prenyl donors. The preferred prenyl acceptor was phlorisobutyrophenone. High transferase activity was also found with phloracetophenone. The product resulting from this acceptor substrate was identified as dimethylallylphloracetophenone in comparison with an authentic sample that

was chemically synthesized. No enzyme activity was found with phloroglucinol as prenyl acceptor.

- Kinetic parameters were determined from Lineweaver-Burk plots. The K_m values were 0.46 mM for DMAPP, 0.52 mM for phlorisobutyrophenone, and 3.8 mM for Fe^{2+}.

- After ultracentrifugation, the majority of enzyme activity (80 %) was found in the supernatant, indicating that the dimethylallyltransferase involved in hyperforin biosynthesis was a soluble enzyme.

- The accumulation of hyperforins in *H. calycinum* cell cultures occurred between day 3 and 6 and was preceded by an increase in dimethylallyltransferase activity. The maximum specific enzyme activity was 3.6 µkat/kg protein.

- The dimethylallyltransferase was purified using ammonium sulphate precipitation, followed by five column chromatographic steps including DEAE and Mono-Q anion exchange chromatography, hydroxylapatite adsorption chromatography, hydrophobic interaction chromatography and gel filtration.

- On a calibrated gel filtration column, the relative molecular mass (M_r) of the dimethylallyltransferase was 63 000. In SDS-PAGE, the purified protein gave two closely spaced polypeptide bands with M_r 31 000 and 31 500, suggesting that the enzyme was active as a heterodimer.

- The purified protein was subjected to two-dimensional electrophoresis, giving four protein spots. Tryptic digestion of the individual polypeptides and sequence analysis of the resulting peptides by ESI-MS/MS revealed that all four polypeptides were isoforms of the elongation factor 2. No sequence similarity was observed with prenyltransferases.

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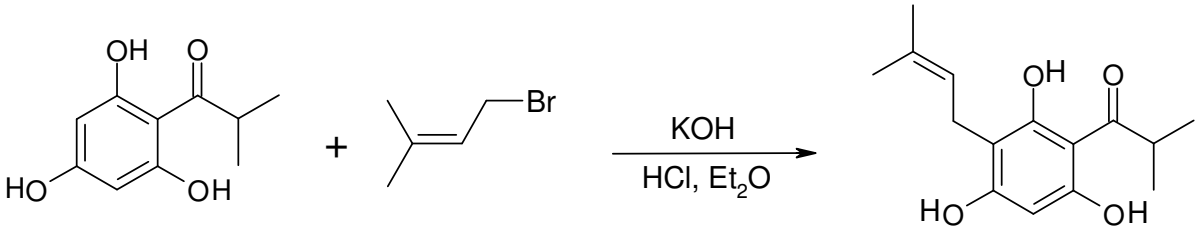
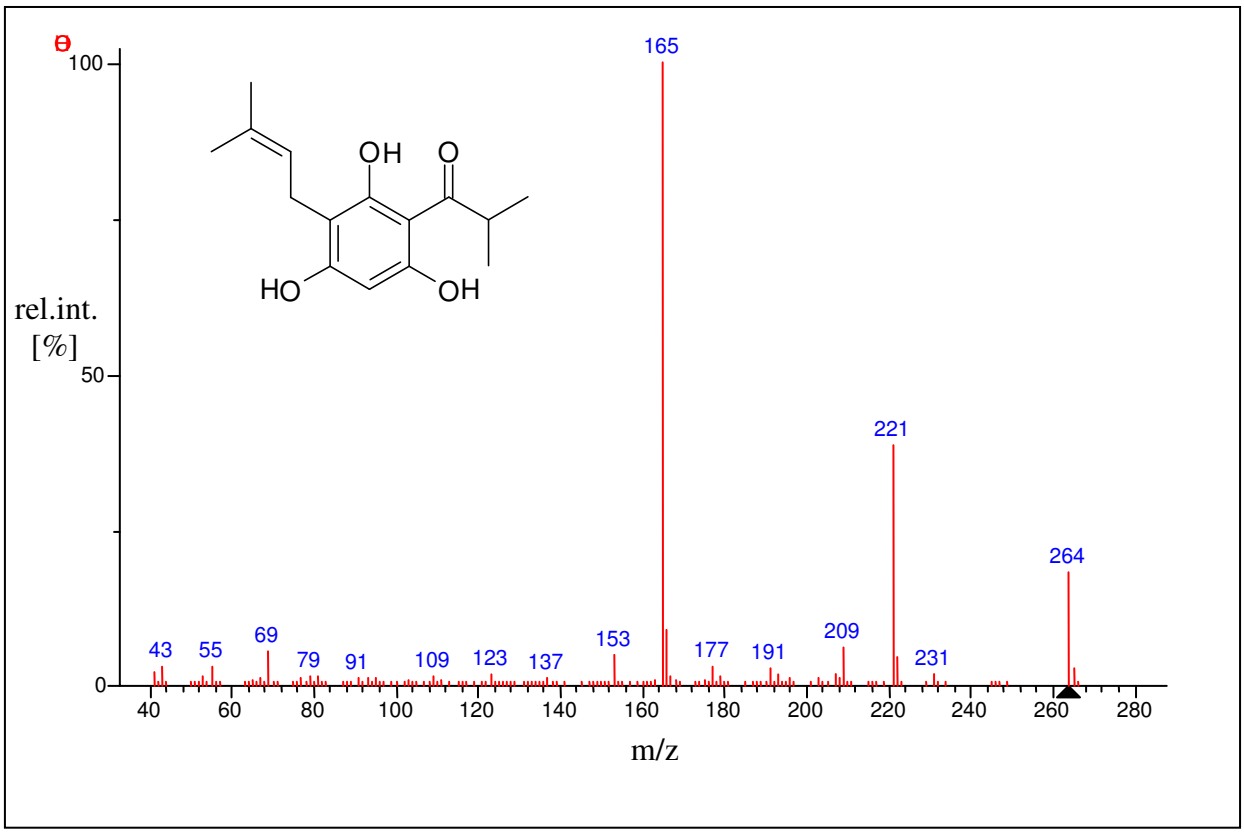
8. Appendices

A) Chemical synthesis of the reference dimethylallylphlorisobutyrophenone

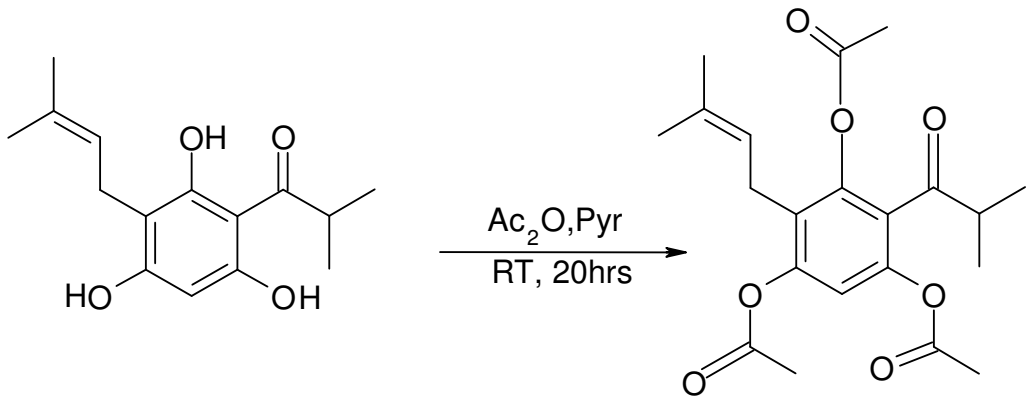


$C_{15}H_{20}O_4$
Exact Mass: 264.07

Mass spectrum of 3-(3-methyl-2-butenyl)-2,4,6-trihydroxyisobutyrophenone:

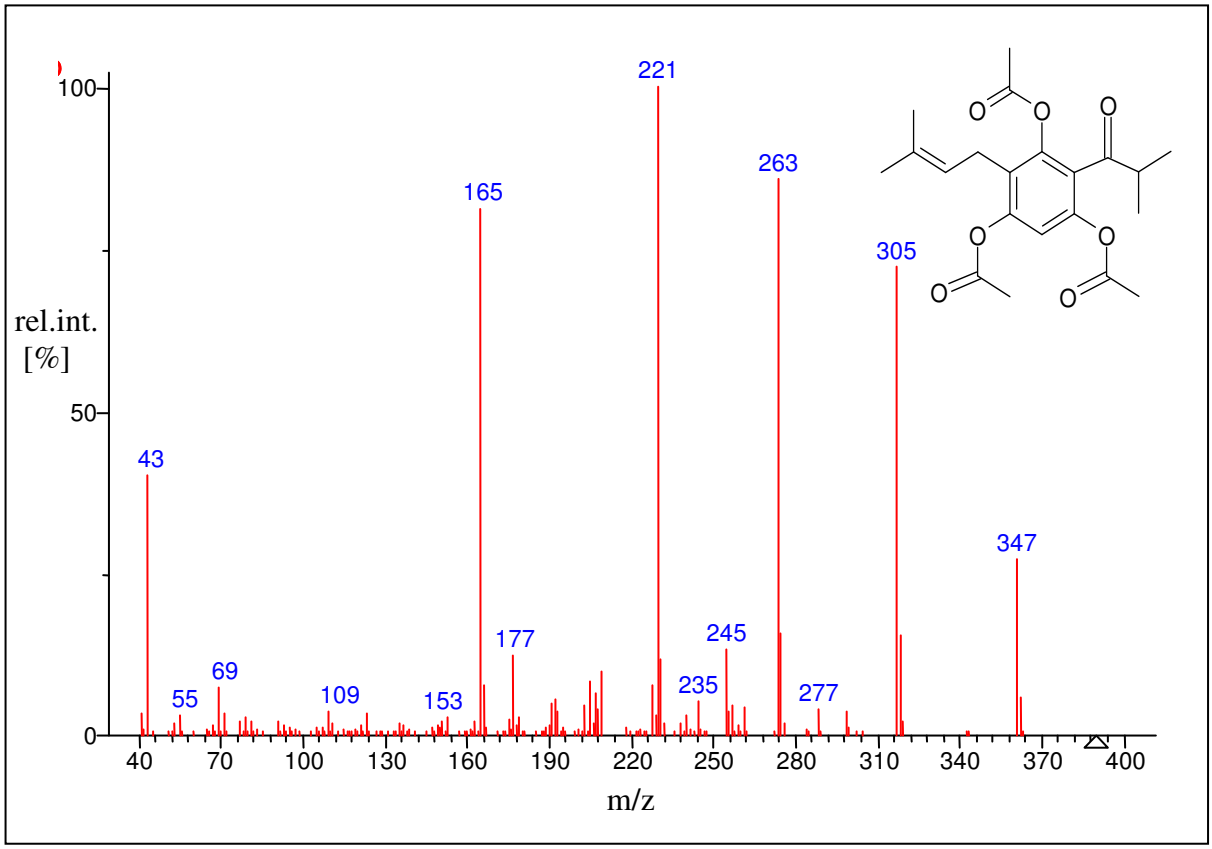


B) Acetylation of the reference dimethylallylphlorisobutyrophenone

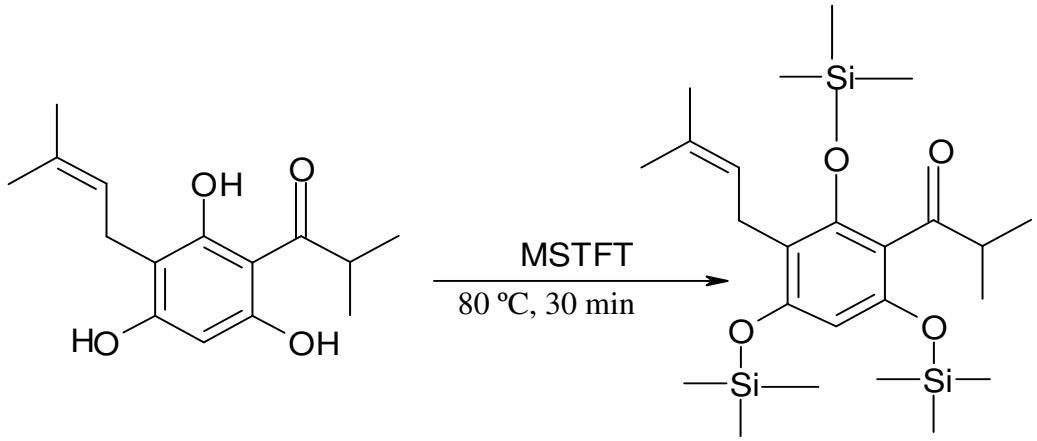


$C_{21}H_{26}O_7$
Exact Mass: 390.04

Mass spectrum of 1- methylpropanoyl-3-[3',3'-dimethylallyl-(1')]-2,4,6-tri-O-acetyl-phloroglucinol:

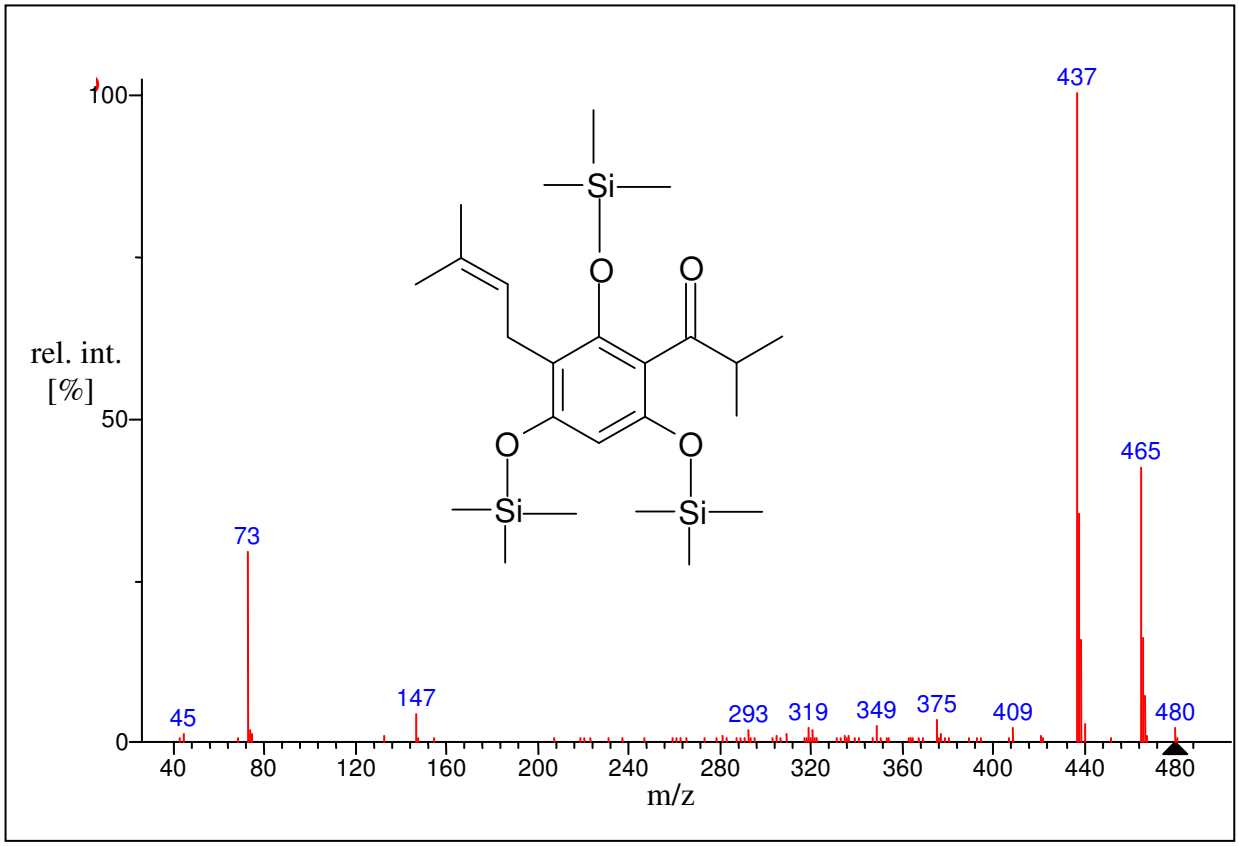


C) Silylation of the reference dimethylallylphlorisobutyrophenone

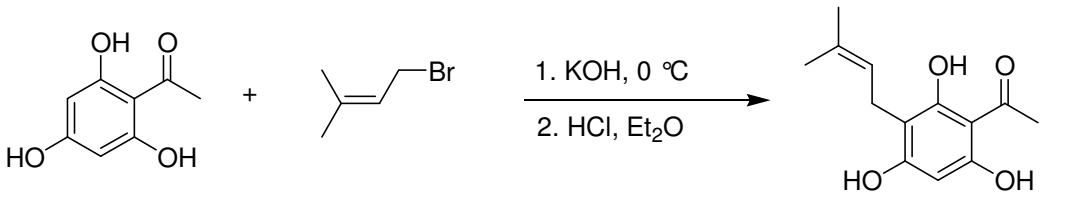


$C_{24}H_{44}O_4Si_3$
Exact Mass: 480,68

Mass spectrum of 2-methyl-1-[3-(3-methyl-but-2-enyl)-2,4,6-tris-trimethylsilanyloxy-phenyl]-propan-1-one:

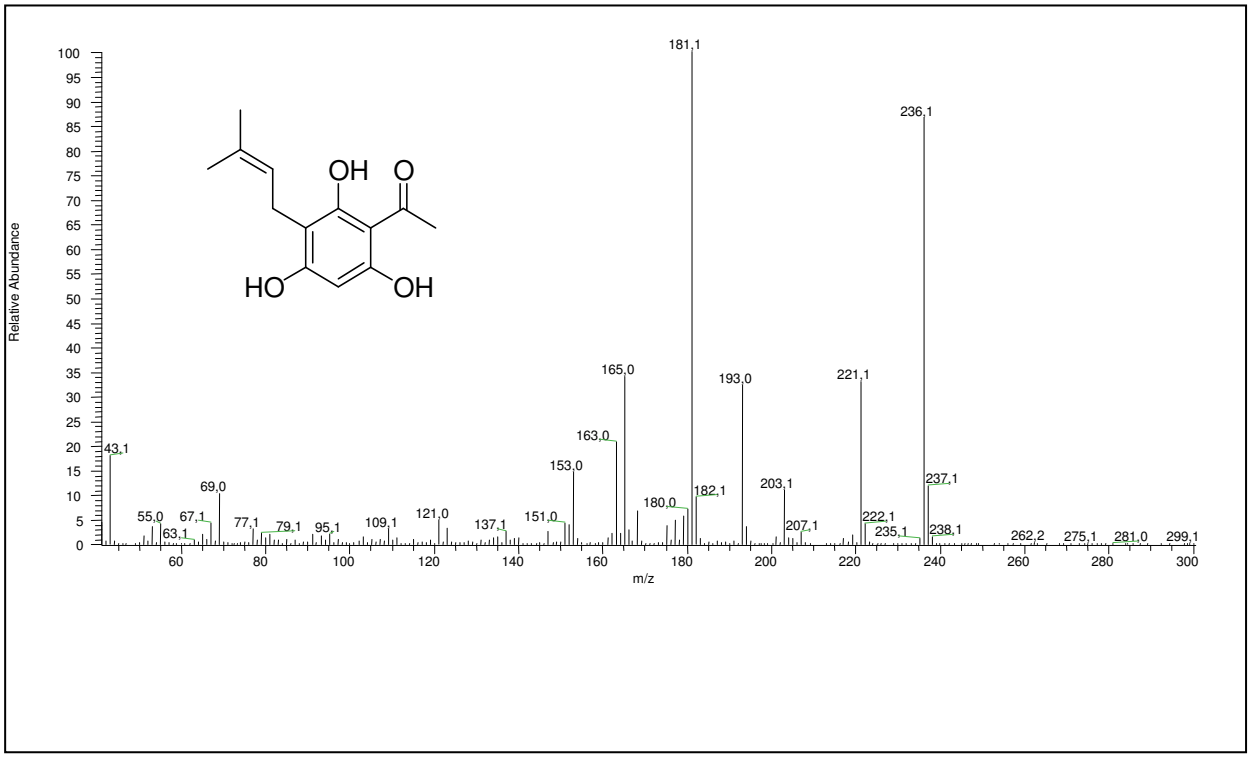


D) Chemical synthesis of the reference dimethylallylphloracetophenone

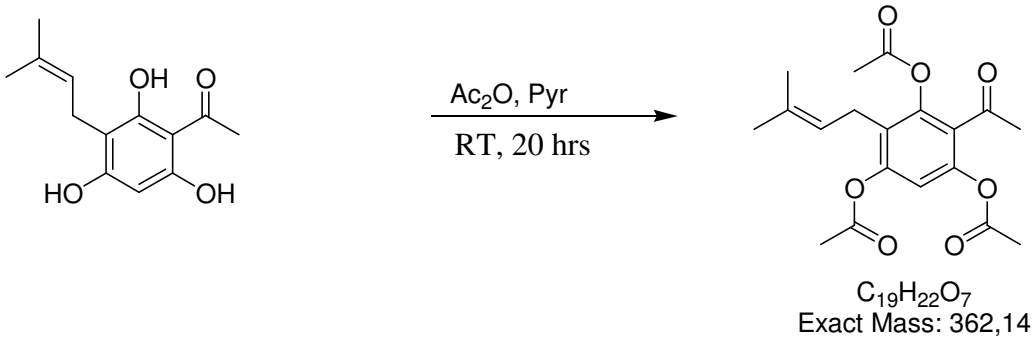


$C_{13}H_{16}O_4$
Exact Mass: 236,10

Mass spectrum of 3-(3-methyl-2-butenyl)-2,4,6-trihydroxyacetophenone:

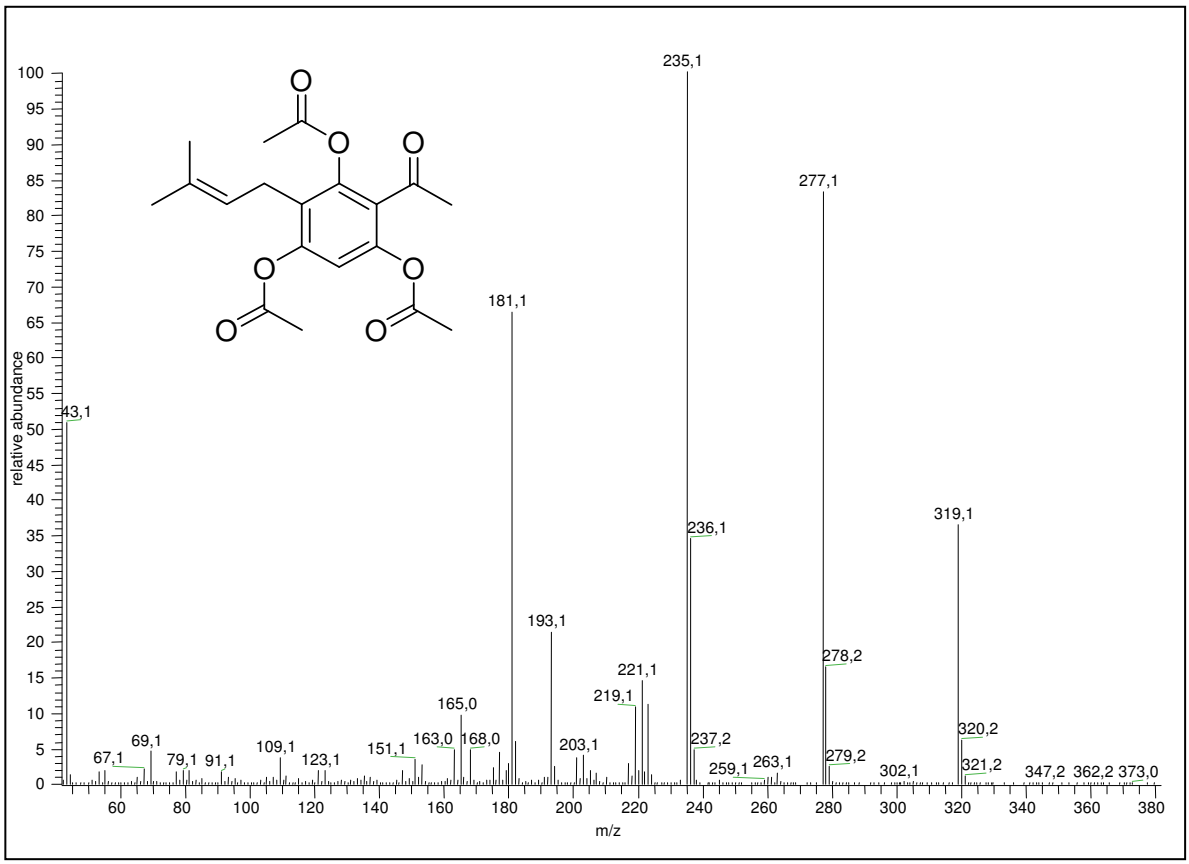


E) Acetylation of the reference dimethylallylphloracetophenone

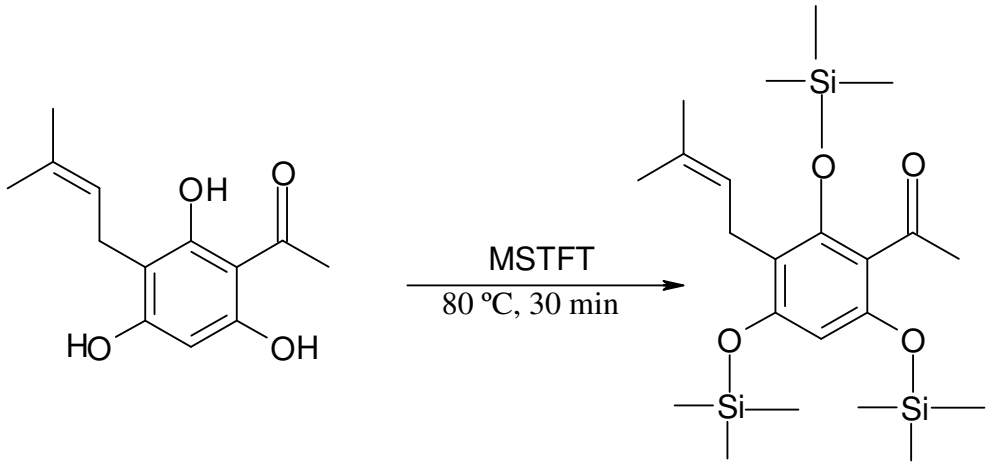


$C_{19}H_{22}O_7$
Exact Mass: 362,14

Mass spectrum of 1-acetyl-3-[3',3'-dimethylallyl-(1')]-2,4,6-tri-O-acetyl-phloroglucinol:

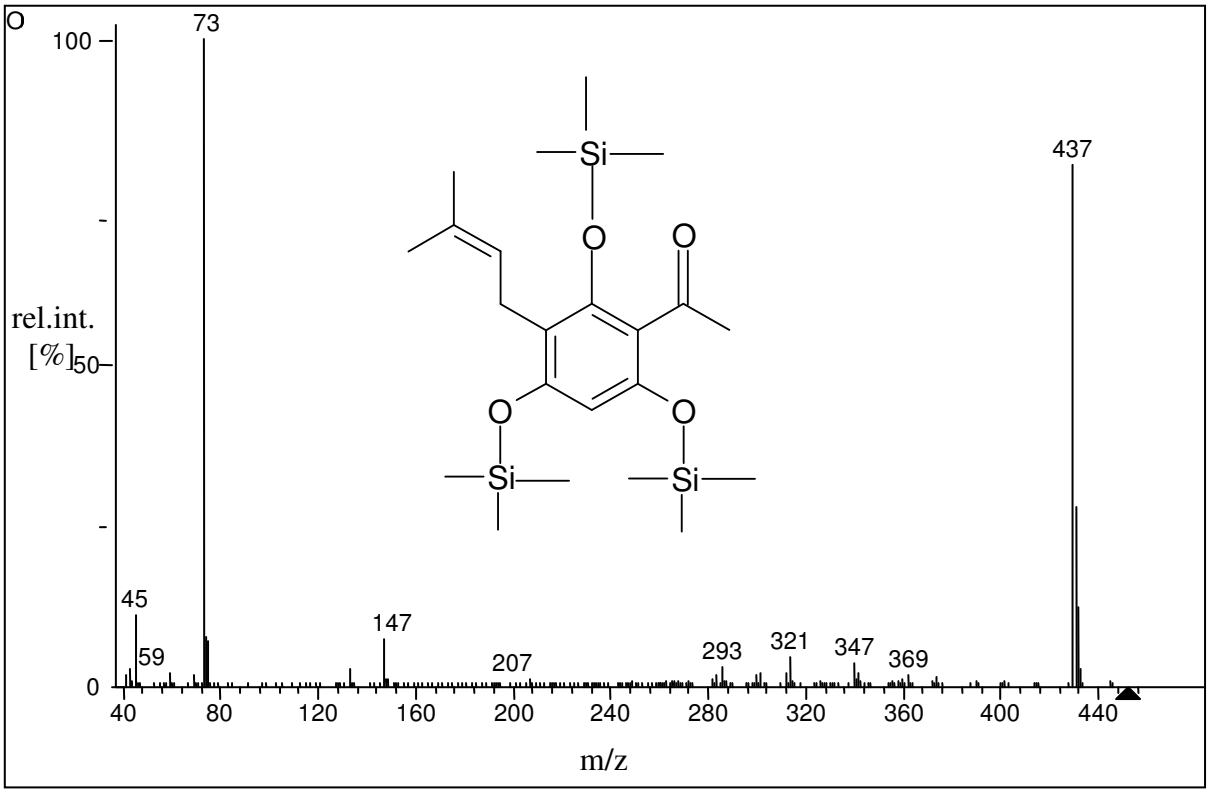


F) Silylation of the reference dimethylallylphloracetophenone



$C_{22}H_{40}O_4Si_3$
Exact Mass: 452.64

Mass spectrum of 1-[3-(3-methyl-but-2-enyl)-2,4,6-tris-trimethylsilanyloxy-phenyl]-ethanone:



Curriculum vitae

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1984 – 1987 Junior High School Al-Dia

1987 – 1990 Senior High School Al-Zahra

Studies: 1990 – 1995 Graduate study at Faculty of Pharmacy, Garyounis University

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Working Experiences:

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11.98 – 05.99 Pharmacist in polyclinical hospital in Benghazi, Libya

06.99 – 12.00 Demonstrator in Pharmaceutical Chemistry, Garyounis University

Language courses:

February 2001 Scholarship from Libyan government for Ph.D. work

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Ph.D. work: 02.02 - 07.05 Institute of Pharmaceutical Biology, Technical University of Braunschweig, Germany